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Our goal is to identify genes involved in the development of the invasive phenotype as these may offer predictive markers and markers of risk of invasive disease in pre-invasive lesions. We have applied our tissue based strategy to directly identify differentially expressed genes between pre-invasive in-situ (DCIS) and adjacent early invasive tumor cell populations and have identified two promising candidate 'invasion' genes. To pursue the role of Psoriasin (S100 A7) we have generated a specific antibody for western blot and immunohistochemistry, transfected a breast cell line to study as a model (MDA-MB-231 ^{psoriasin}), and determined that relatively higher psoriasin mRNA and protein correlates with the more invasive ER negative phenotype, within the range seen in invasive tumors. Lumican is a small leucine-rich proteoglycan, overexpressed by fibroblast-like cells, particularly immediately adjacent to in-situ elements , and at higher levels in association with several parameters indicative of more biologically aggressive breast tumors. It is also the most abundant proteoglycan within its stromal gene family. The pattern of upregulation and abnormal protein deposition within subregions of breast tumors, and its known role in cross linking of collagen in normal tissues, supports the hypothesis that changes in lumican expression may influence invasiveness.

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P. H. Watson — 18th Sept '95

PI - Signature — Date

TABLE OF CONTENTS

INTRODUCTION.

The acquisition of the ability to invade is the single most important aspect of breast tumor progression, as it is a crucial component of the process of metastasis and marks the transition from local to life threatening disease. Assessment of risk of progression to invasive breast cancer has recently become an increasingly recognizable and defined problem in clinical management[1]. This is partly due to the increasing number of patients who present with pre-invasive ductal carcinoma in-situ, (DCIS)[2]. More recently, the demonstration that breast cancer can be delayed or inhibited by tamoxifen therapy in women at high risk as defined in the NSABP trial[3] has also provided a new focus and impetus to improve the accuracy of risk determination.

The management of DCIS depends on the estimation of the likelihood of recurrence as in-situ or invasive disease. Recent morphological studies have provided useful potential improvements to older classifications of pre-invasive disease with more accurate prognostic significance[4], however there is clearly a need for better predicators of biological potential[5]. But although there has been an impressive growth in the knowledge of genes involved in tumor invasion and metastasis[6], the importance of most of these factors in the development of the invasive phenotype in the complex heterogeneous disease of human breast cancer has been difficult to establish[7,8]. This is partly because most of these genes (including cell adhesion molecules, proteases and motility factors) were first identified in tumor cell lines and systems other than epithelial breast carcinoma. This in turn reflects a lack of suitable breast specific models or breast cell lines that are representative of the pre-invasive, in-situ stage of cancer.

Invasion is likely to involve many gene alterations which may arise individually during much earlier stages of tumorigenesis without causing the invasive phenotype, and the sum of many changes probably culminates in the lesion we recognize as invasive cancer. Genes that can influence invasion may therefore show changes at earlier stages than DCIS and may offer markers to define risk. To address this critical issue in the biology of early breast tumor progression, many groups have surveyed DCIS lesions with microsatellite markers in search of regions of LOH that

may harbor tumor invasion suppressor genes. This approach has yielded some interesting loci but not specific genes as yet[9]. We have developed an alternative, novel approach in our laboratory to identify genes and alterations that may contribute to the invasive phenotype in-vivo. This approach has required technical development to enable microdissection of histologically defined microscopic components within single breast tumor sections[10] and adaptation of established subtraction hybridization techniques to accept small input RNA sample[11] and has been crucially dependent on the design and maturation of the tumor bank resource for histologically defined frozen breast tissues[12].

It is important to appreciate that not all candidate loci that show LOH or genes that show alteration of expression in an individual tumor will be relevant to the invasive phenotype. Altered expression may be; irrelevant to invasion but occur as a result of a unique genetic event; linked to an adjacent gene involved in invasion and altered as a result of a common genetic event; directly involved in a unique aspect of the process of invasion in a single case, or directly involved in a conserved aspect of the invasion process. It is therefore important to distinguish these possibilities and to establish the relevance to the complex process of invasion by further laboratory and correlative studies.

Our specific hypothesis is that alteration of gene expression is responsible for the progression of DCIS to invasive breast cancer and the acquisition of the invasive phenotype. Our specific technical objectives are;

1.To identify and clone differentially expressed genes that may contribute to the invasive phenotype in-vivo. Novel microdissection and subtraction hybridization techniques will be used to directly compare gene expression between DCIS and adjacent early invasive ductal carcinoma cell populations within the same tumor specimen.

2. To study the role in breast cancer progression of altered expression of the candidate 'invasion genes' that have been identified in specific aim 1 by assessment of pattern of expression in-vivo and by manipulation of expression in breast cell lines.

BODY.

TECHNICAL OBJECTIVE 1. Initial review of 388 invasive ductal carcinomas in the Tumor Bank found 32% contained an identifiable in-situ component but less than 5% were suitable for microdissection. These cases comprised early invasive cancer with >50% in-situ and associated invasive components, microdissectable within a single tissue block and with similar cytology and stromal backgrounds. Prior to this grant we had completed analysis of one case (tumor $^{dcis/inv}$ #1) and identified one gene, psoriasin S100A7, as differentially expressed between in-situ and invasive component[13]. During the past year of this grant we have applied microdissection, subtractive hybridization, cDNA cloning and analysis to complete a second case experiment (tumor $^{dcis/inv}$ #2). In this we have identified cDNA's corresponding to 15 known genes, 5 ribosomal genes, and 4 unknown expressed sequences apparently overexpressed in either DCIS or invasive components (Table 1).

To rapidly determine the potential of this approach we have initially focused on 6 known cDNAs (identified in other systems) from the two cases analyzed. We have confirmed amongst these, real differential expression (3 cDNA's, see below), differential expression attributable to differences in local regional composition (Ets-like cDNA expressed in the denser vascular endothelium of invasive component in the sentinel case) and false positives (2 cDNA's, tra-1 and OCP2) by in-situ hybridization and RT-PCR. Consideration of the patterns of expression amongst the 3 differentially expressed cDNA's has encouraged us to pursue the potential role of psoriasin, lumican and mammaglobin genes in invasion (see below).

However, we have also identified a new problem with the subtraction assay that we have used. We have dissected 2 additional tumors and performed subtraction assay using the extracted mRNA (tumor $^{dcis/inv}$ #3 and #4), however in both cases the GAPDH control lanes showed no significant subtraction[11], suggesting a problem with non-specific retention of cDNA on newer batches of magnetic Dynal beads. The problem has persisted despite repetition with alternative sources of beads, new beads from Dynal, and discussion with the company, and remains unresolved. In the

meantime we have begun to test an alternative assay to compare cDNA's from small microdissected tissue samples[14]. We have performed RDA on small amounts of RNA extracted from MDA-MB-231 cells and compared parental cells with cells transfected with psoriasin, and we are currently analyzing the multiple cDNA's that are apparently differentially expressed as a result of cloning or differential expression.

TECHNICAL OBJECTIVE 2. We have pursued 3 potential 'invasion' genes, psoriasin, lumican and mammaglobin as outlined below.

Psoriasin (S100A7). We have previously shown that psoriasin expression is altered in association with early tumor progression[13] and from what is known of the function of psoriasin and other S100 genes we believe that it is important to pursue the role of psoriasin in invasion[15]. Psoriasin has been shown to have an in-vitro effect as a secreted chemotactic factor for leucocytes[15] and we can speculate that this gene might influence tumor progression indirectly through modulation of the host immune response or directly through influence on epithelial cell motility[16]. Analogy with other closely related S100 proteins suggests that interaction with intracellular proteins and processes[17] should also be considered.

To pursue our studies we initially requested anti-psoriasin ab made against recombinant psoriasin from Dr Cellis, Arhus, Denmark[15], who kindly provided us with a small sample which we have tested by Western blot and immunohistochemistry. Our experience is that this ab works on Western blot but in our hands does not work on immunohistochemistry applied to frozen or paraffin tissues. Given potential cross reactivity with other s100 abs and inability to use this as a tool to examine paraffin embedded breast tissue samples we decided to generate a new antibody (while conserving the Arhus antibody for analysis of transfected cells and comparison with new antibodies). In the past few months we have successfully developed a psoriasin specific chicken IgY polyclonal antibody (with Bionostics/Aves Labs) through immunization of chicken with a 13aa COOH terminal peptide (that shows low homology to other S100 proteins). We have

confirmed specificity by 2D electrophoresis and Western blot[18] and conducted parallel experiments using the Arhus anti-psoriasin ab and a pan-anti-S100 antibody from Sigma to demonstrate that this anti-psoriasin IgY works well on Western blot. Preliminary experiments also suggest that it will work by immunohistochemistry.

We have now completed a study[18] based on combined Western blot, RT-PCR and in-situ hybridization analysis to determine that psoriasin protein and mRNA expression correlate well in tumors, and that higher levels of psoriasin expression (within the range of low levels seen in invasive tumors) correlates with the ER negative phenotype (Spearman test $r= -0.673$, $p<0.0001$, $n=58$) and that psoriasin levels are lower in certain special invasive types of tumors, including tubular and colloid ($n=12$)[18]. We will now pursue this observation and the relation to risk of recurrence in DCIS lesions by immunohistochemistry and also address the following questions with respect to its functional effect on invasion and early tumor progression, its potential role as a marker of risk of progression to invasive disease, and its functional effect in the breast epithelial cell[16].

We are also studying the functional significance of altered expression of psoriasin by manipulation of expression in human breast cells. One avenue we have taken is to examine whether psoriasin can inhibit invasion in invasive tumor cells, overexpressing psoriasin by stable transfection of a CMV-psoriasin construct into a specific subline of MDA-MB-231 breast cells that do not express psoriasin and that are known to be invasive , tumorigenic and metastatic in an in-vivo metastasis model in our own hands[19] and that have previously been shown to be amenable to genetic modulation of their metastatic properties[19]. We have isolated 2 MDA-231^{psoriasin} clones that demonstrate high and low expression determined by Northern and Western Blot[18] and we are currently studying the behavior of these cells using in-vitro Boyden chamber invasion assays and in-vivo in a balb-c nude mouse model. Another avenue we have taken is to examine whether psoriasin can influence early progression and invasiveness in 'normal' cells. Accepting the limitations of several potential models we have focused on the MCF10AT model[20] and have

transfected CMV-psoriasin into MCF-10AT3B cells derived from non-neoplastic breast tissues which do not express psoriasin. The latter cells have previously been manipulated by introduction of H-ras and passage in xenografts. We are currently screening zeocin resistant clones for psoriasin expression prior to in-vitro assessment of growth and invasiveness and in-vivo study of the effect of psoriasin on early tumorigenesis and progression.

Lumican. The development and progression of breast carcinoma is caused by complex alterations in multiple genes and cellular components, including altered interactions between the epithelial and stromal cells, which are manifested in tumors by the stromal reaction[21]. The breast stroma includes a variety of different types of fibroblast and a range of proteins, glycoproteins and proteoglycans which may play a role in this aspect of tumor biology. We have recently extended this list by identifying lumican[22], a member of the small leucine-rich proteoglycans (SLRP's)[23] as an mRNA that is overexpressed in the in-situ component of tumor $^{dcis/inv}$ #2[24]. In-situ-hybridization studies were subsequently performed on the sentinal tumor and a panel of 30 additional invasive carcinomas. From these studies it has become clear that lumican is expressed by stromal fibroblast-like genes, that it is particularly overexpressed by fibroblast-like cells immediately adjacent to in-situ elements (explaining the fact that we picked this gene up when trying to dissect in-situ and invasive components), and that higher levels of expression are associated with several parameters indicative of more biologically aggressive tumors[24] (including high grade, low ER and younger patient age). Our subsequent very recently completed work[25] based on 45 normal tissues and selected types of tumors confirms that lumican protein is uniformly highly abundant relative to other members of the family of small leucine-rich proteoglycans (SLRP's) including decorin, biglycan and fibromodulin. Furthermore, combined analysis by in-situ hybridization, RT-PCR, Western Blot suggests that this pattern of upregulation is distinct from the closely related decorin gene. Microdissection studies also show that lumican expression in tumors may be associated with abnormal distribution of lumican protein within the stroma manifested by discordance between mRNA and protein deposition within subregions of breast tumors. It is possible to speculate that these changes in lumican and decorin expression may represent a positive host response to

abrogate the disorganization of collagen within the tumor stroma[27] and inhibit the growth of epithelial cancer cells through the increased availability of inhibitory growth factors[27]. Alternatively, induction of lumican may represent a feedback response, to the negative effects of altered deposition of the lumican protein and the resulting disorganization of the collagenous stroma that facilitates tumor cell invasion. Similarly, decrease in decorin may remove an inhibitory effect on epithelial tumor cell growth though repression of p21[28]. A role for and the distinction between these opposing potential effects will clearly require further study of the role of lumican in invasion. We will initially pursue the following directions. We will transfect human lumican and decorin cDNA's into an immortalized murine mammary fibroblast line and study the effect of this on fibroblast function and neoplastic syngeneic carcinoma cells utilizing both in-vitro 3-D co-culture, growth and invasion assays, and in-vivo study of mixed fibroblast and tumor populations (in collaboration with Dr C Roskelley, UBC). We will also pursue the factors that influence upregulation of lumican and altered distribution within tumor stroma. This will be through co-culture experiments utilizing human and murine fibroblast and epithelial cell lines to identify the necessary interactions for lumican upregulation (in collaboration with Dr P Roughley, McGill. Continuation of our in-vivo human studies will involve assessment of the precise relationship between lumican protein disposition and other proteoglycans.

Mammaglobin. This gene has recently been identified by others as a mammary specific gene expressed by approximately 2/3 of invasive breast tumors[29]. Mammaglobin is considered to be a member of the uteroglobin gene family and also maps to a region, 11q13 that is frequently associated with alterations in breast tumorigenesis[30,9]. However the cellular origin of expression of mammaglobin and the relationship between expression and tumor progression has not been previously determined. We identified mammaglobin as a gene that is differentially overexpressed in the in-situ versus the invasive component of the initial microdissection case (tumor $^{dcis/inv}$ #2) [31]. However, our subsequent results show that the pattern of mammaglobin expression within breast tissues and tumor components is complex. In-situ hybridization analysis of 13 primary tumors containing normal, in-situ, invasive elements revealed that mammaglobin expression occurs in all

compartments and can overexpressed in either the in-situ or invasive component. However, it is restricted to epithelial cells, and persists in axillary lymph node metastases. Reverse transcription-PCR analysis of 20 tumors and matched lymph nodes showed a direct correlation between mammaglobin mRNA expression and histological detection of nodal metastases[32]. So while this data suggests that mammaglobin is unlikely to be relevant to invasion, our results suggest that mammaglobin could be a marker of axillary lymph node breast metastases.

CONCLUSION

Elucidation of the molecular changes involved in the development of the invasive phenotype is a critical clinical question because the lethal effects of distant metastasis can only occur after the onset of invasive capability and the ability to assess these genetic alterations may offer markers of risk of invasive disease in pre-invasive lesions. We have identified two genes potentially involved in breast cancer cell invasion. Psoriasin (S100 A7), a member of the S100 calcium binding protein family is differentially expressed in pre-invasive DCIS vs invasive components and expression within different types of invasive carcinoma is related to estrogen receptor status and indices of differentiation. Thus the overall pattern of expression and its known capability to influence cell motility is compatible with the hypothesis that altered expression may be a marker of invasiveness. Lumican is a small leucine-rich proteoglycan, that we have found overexpressed in breast cancer and is the most abundant proteoglycan within its stromal gene family. The pattern of upregulation of lumican is distinct from the related decorin gene, has a distinct regional distribution within tumors adjacent to pre-invasive elements and the invasive margin, and shows abnormal protein deposition within subregions of breast tumors. Although not previously studied in tumor progression, its known role in cross linking of collagen supports the hypothesis that changes in lumican expression may influence invasiveness. Further studies to microdissect and analyze pre-invasive and invasive tumor components and to examine the role of these genes in invasion through manipulation of cell models are in progress.

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APPENDICES

Appendix A) Table 1: Tumor case #2, cDNA's apparently differentially expressed between tumor components.

Appendix B) Watson PH, Leygue E, Murphy LC. "Molecules in focus- psoriasin (S100A7)" Int J Biochem Cell Biol. 30(5): 567-571. 1998

Appendix C) Watson PH, Hiller T, Snell LS, Murphy LC, Leygue E, Dotzlaw H, Kossakowska A, Kulakowski A. "Update to : microdissection RT-PCR analysis of gene expression in pathologically defined frozen tissue sections" in "the PCR technique: RT-PCR, editor Paul Siebert, Eaton Publishing.

Appendix D) Al-Haddad S, Zi Yuan, Huang A., Leygue E., Hiller T., Snell L., Dotzlaw, H, Murphy L., Watson P. Psoriasin(S100A7) expression in human breast carcinoma. Manuscript in preparation

Appendix E) Leygue E, Snell L, Dotzlaw H, Hole K, Hiller T, Roughley P, Watson P, Murphy L, "Expression of Lumican in human breast carcinoma" Cancer Research 58(7): 1348-1352, 1998.

Appendix F) Leygue E, Snell L, Dotzlaw H, Hole K, Hiller-Hitchcock T, Murphy LC, Roughley PJ, Watson P,. "Lumican and decorin are differentially expressed in human breast carcinoma" submitted to Am J Pathology, Sept 1998

Appendix G) Leygue E, Snell L, Dotzlaw H, Hole K, Hiller T, Roughley P, Watson P, Murphy L, "Mammaglobin, a potential marker of breast micrometastasis" submitted to J Path July 1998

APPENDIX A

Table 1.

Tumor case #2, cDNA's apparently overexpressed in the DCIS > Invasive component.

Clone #	Insert Length	Sequence Blasted	Homology	Bank	Reference #	Name (best score)
TIS3#3-3	675bp	82bp	poor 63/78	genbank dbEST	S75844S4 R74799	mouse IL2 receptor γ chain mouse similar glyoxylate - induced protein (E.coli)
TIS3#3-4	675bp	106bp	88/89	genbank	HSACTVBA3	activin β-A subunit
TIS3#4-2	650bp	133bp 133bp	poor 121/128	genbank dbEST	HUMPKD1GEN H66659	similar to SP: TRLC_DROME P36951
TIS3#5-3	600bp	76bp	poor poor	genbank dbEST	HSU67615 R99645	beige protein homolog
TIS3#6-1	550bp	69bp	poor 31/31	genbank dbEST	A28847 R71014	cDNA clone
TIS3#6-3	550bp	113bp	poor 113/113	genbank dbEST	CEF43D2 HSC2VF072	cDNA clone
TIS3#7-1	500bp	164bp	split 99/104 & 52/53	genbank	HSU79254	clone 23693 mRNA
TIS3#8-1	450bp	112bp	110/112	genbank	HUMEF1AA	elongation factor 1-α
TIS3#8-4	450bp	120bp	43/47& 21/23 119/120	genbank dbEST	MUSRPLPSC AA074860	mouse ribosomal protein L30 similar to L30 (Strat.ovarian cancer)
TIS3#9-1	400bp	181bp	177/181	genbank	HSU21128	lumican
TIS3#9-6	400bp	123bp	poor 68/69& 64/65	genbank dbEST	HIVU51189 AA173423	Strat. ovarian cancer
TIS3#10-1	375bp	115bp	poor 114/115	genbank dbEST	ASNALDAA AA035073	Soares pregnant uterus
TIS3#11-9	1kb	214bp	203/207	genbank	HSTROISOA	tropomyosin isoform
TIS3#12-4	900bp	97bp	good	genbank		numerous & varied
TIS3#14-1	700bp	26bp	25/26	genbank	HUML12A	ribosomal protein L12
TIS3#15-1	650bp 131bp		poor 128/130	genbank dbEST	AA166778	Strat. ovarian cancer
TIS3#16-1	575bp	61bp	60/61	genbank	HSRP26AA	ribosomal protein L26
TIS3#17-2	550bp	48bp	?	genbank	HUMCH13C3A	ribosomal protein S3a
TIS3#17-5	550bp	51bp	51/51	genbank	HSU33147	mammaglobin
TIS3#18-1	500bp			genbank		ribosomal protein L23a
TIS3#19-4	450bp			genbank		M phase phosphoproteins
TIS3#19D*	450bp	45bp	45/45	genbank	HSTRA1	tra1 mRNA
TIS3#21-1 TIS3#21-4	425bp	42bp 124bp	41/42 122/122	genbank genbank	HSU14394	tissue inhibitor of metalloproteinases (TIMP3) also reverse

Table 1.

Tumor case #2, cDNA's apparently overexpressed in the Invasive > DCIS component.

Clone #	Insert Length	Sequence Blasted	Homology	Bank	Reference #	Name (best score)
TIV3#3-2	720bp	92bp	88/90	genbank	HUMMGPA	matrix Gla protein
TIV3#3A	720bp	50bp	49/50	genbank	HUMGBP2	guanylate binding protein isoform II
TIV3#4-2	700bp	78bp	28/32& 27/31& 16/17	genbank	RATETSONCA	rat Ets-1
TIV3#4C	700bp	81bp	60/65 80/81	genbank dbEST	RATONC T29955	rat mRNA expressed in embryo and tumor similar to single stranded binding protein P9
TIV3#5E	600bp	98bp	52/54& 43/45	genbank	HSATPCP1	mitochondrial ATP synthase c subunit
TIV3#5I	600bp	109bp	74/76& 33/33	genbank	D89667	c-myc binding protein MM-1
TIV3#6-1	550bp	134bp	poor	genbank	CHKP105A	chicken p105
TIV3#7A	500bp	120bp	102/114 118/120	genbank dbEST	D86344 MUSMA3CDNA AA129597	mouse topoisomerase inhibitor mouse MA-3 (apoptosis related) Soares pregnant uterus
TIV3#7B	500bp	120bp	41/50& 20/24 116/120	genbank dbEST	MUSMA3CDNA D86344 H16115/ AA081192	mouse (see 7A) cDNA clone/endothelial cell cDNA
TIV3#8-3	475bp	48bp	poor 28/28& 28/37	genbank dbEST	HSU33760 C05052	cyclin A/CDK2 assoc.p19 heart cDNA
TIV3#8D	475bp	113bp	111/112 111/112	genbank	HSU33760 HSU37558	cyclin A/CDK2 assoc.p19 OCP2 mRNA
TIV3#9-5	465bp	107bp	58/69& 45/51	genbank	HSEF1AC	elongation factor 1-α
TIV3#9A	465bp	57bp	57/57	genbank	HSCOL3A1	pro-α1 type III collagen
TIV3#10-1	450bp	43bp	43/43 43/43	genbank	MUS13KMP MUSLBP	mouse 13K protein mouse lipid binding protein
TIV3#13-2	415bp	86bp 47bp	poor 47/47	genbank dbEST	CELC25B8 C16158	cDNA clone - aorta
TIV3#14-1	400bp	77bp 40bp	 40/40	genbank dbEST	CLEGCGA HSAAAEFZZ	
TIV3#14-5	400bp	76bp 39bp	poor 39/39	genbank dbEST	MTLB2 R71224	cDNA clone

APPENDIX B

PERGAMON

The International Journal of Biochemistry & Cell Biology 30 (1998) 567–571

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Molecules in focus

Psoriasin (S100A7)

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Abstract

Psoriasin (S100A7) is a relatively new member of the S100 gene family that is located within the S100 gene cluster on chromosome 1q21 and shares the typical calcium binding domains that define this family of proteins. It was first identified as a 11.4 kDa cytoplasmic and secreted protein isolated from skin involved by psoriasis, which can be induced in cultured squamous epithelial cells. It is now known to be expressed by both normal cultured and malignant keratinocytes and malignant breast epithelial cells within ductal carcinoma *in situ*, suggesting an association with abnormal pathways of differentiation. Current evidence supports a role in the pathogenesis of inflammatory skin disease, as a chemotactic factor for hematopoietic cells, and a role in early stages of breast tumor progression in association with the development of the invasive phenotype. While therapeutic potential as a target for modulation of the inflammatory response in psoriasis awaits further studies, potential clinical applications already include a role as a detection marker for squamous cell carcinoma and a diagnostic marker to distinguish *in situ* from invasive breast cancer cells. © 1998 Elsevier Science Ltd. All rights reserved.

Keywords: Psoriasin; S100; Chemotaxis; Invasion; Breast cancer; Bladder cancer

1. Introduction

Psoriasin is a relatively new member of the S100 gene family that was first identified by [9] as a 11.4 kDa protein induced in squamous epithelial cells of the epidermis isolated from skin involved by psoriasis. Psoriasin shares homology and chromosomal proximity with other members of the S100 gene family, justifying classification as S100A7. The S100 genes encode small cytoplasmic and secreted proteins that share EF-hand helix-loop-helix domains that are important for their function as calcium binding proteins [14]. Broad roles have been proposed for these genes in cell growth, differentiation and determination of cell shape.

2. Structure

The Psoriasin gene maps to chromosome 1q21.2-q22, within a region that encompasses at least 12 of the S100 gene family (see Fig. 1) and several other epidermal differentiation genes [1, 13]. Although the gene has not been sequenced it is likely that it conforms to the organization of adjacent S100 genes. In most

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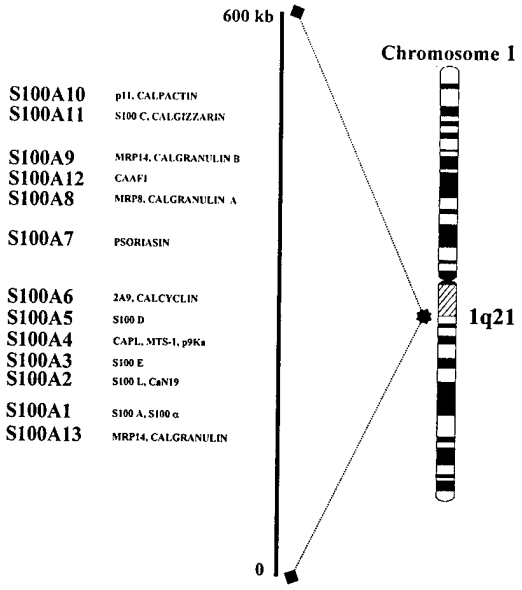

Fig. 1. Diagram of physical map of the S100 gene cluster on chromosome 1q21. Current nomenclature and previous names/synonyms are given on the right and genes are ordered by approximate relative locations.

cases, these possess a simple three exon/two intron structure, with the first exon contributing most of the 5′ untranslated region to the mRNA and the second and third exons covering the coding sequences and contributing one of the two EF-hand-like calcium binding motifs that characterize these proteins. The Psoriasin protein includes an *N*-terminal calcium binding signature that lacks the spacing provided by an additional three amino acids when compared to other S100 genes and a canonical *C*-terminal EF-hand motif. It shares closest overall homologies at the amino acid level to two newly described S100 genes [4], bovine CAAF2 (77%) and CAAF1/S100A12 (63%), as well as recognizable association with MRP8/S100A8 (32%) and p11/S100A10 (23%).

3. Biological function

Psoriasin expression is restricted to keratinocytes and breast epithelial cells, in contrast to the overlapping pattern of the related subgroup of S100 genes A8, A9 and A12, which are also expressed in hematopoietic cells of myeloid lineage. It is known to be both a cytoplasmic and secreted protein that is upregulated in keratinocytes in response to calcium and retinoic acid and during abnormal pathways of differentiation in culture, where Psoriasin is amongst the most highly induced proteins [5]. While the normal function of this protein is unclear it might be deduced from its close relatives. The S100 proteins are believed to influence calcium mediated signal transduction and cellular events through direct target protein interactions, as opposed to a function as mere storage buffers. In this capacity, targets for related S100 proteins include intermediate filaments (S100A8) and annexin (S100A10), implying a role in modulation of cell shape/cytoskeletal architecture or intracellular signal transduction [14]. Heterodimers of the S100A8 and A9 proteins may also have an intracellular activity (as a calcium regulator and/or inhibitor of casein kinase activity) in addition to a complex extracellular activity, both as a migration inhibition factor and a chemotactic factor for monocytes and granulocytes under different circumstances [3]. In the case of Psoriasin, the association with psoriasiform hyperplasia of the skin and its known regulation suggests a role in keratinocyte differentiation. However, Psoriasin may also act as a chemotactic agent [6] to stimulate the neutrophil and $CD4^+$ T lymphocyte infiltration of the epidermis that characterizes psoriasis. These putative functions are summarized in Fig. 2.

4. Role in pathology

The monogram Psoriasin endorses its association with psoriasis and high levels of expression in psoriasiform epidermal hyperplasia adjacent to traumatic skin ulcers have also been observed. However, the gene is clearly also expressed under conditions of abnormal epithelial differentiation and is secreted by neoplastic keratinocytes in carcinoma of the bladder [2]. More recently, Psoriasin and the related S100 A8 and A9 pro-

teins have also been found in other abnormal epithelia, including neoplastic breast ductal epithelium and bronchial epithelium in individuals with cystic fibrosis.

5. Role in breast cancer

Disruption of calcium signalling pathways has been implicated as a central mechanism in tumor-

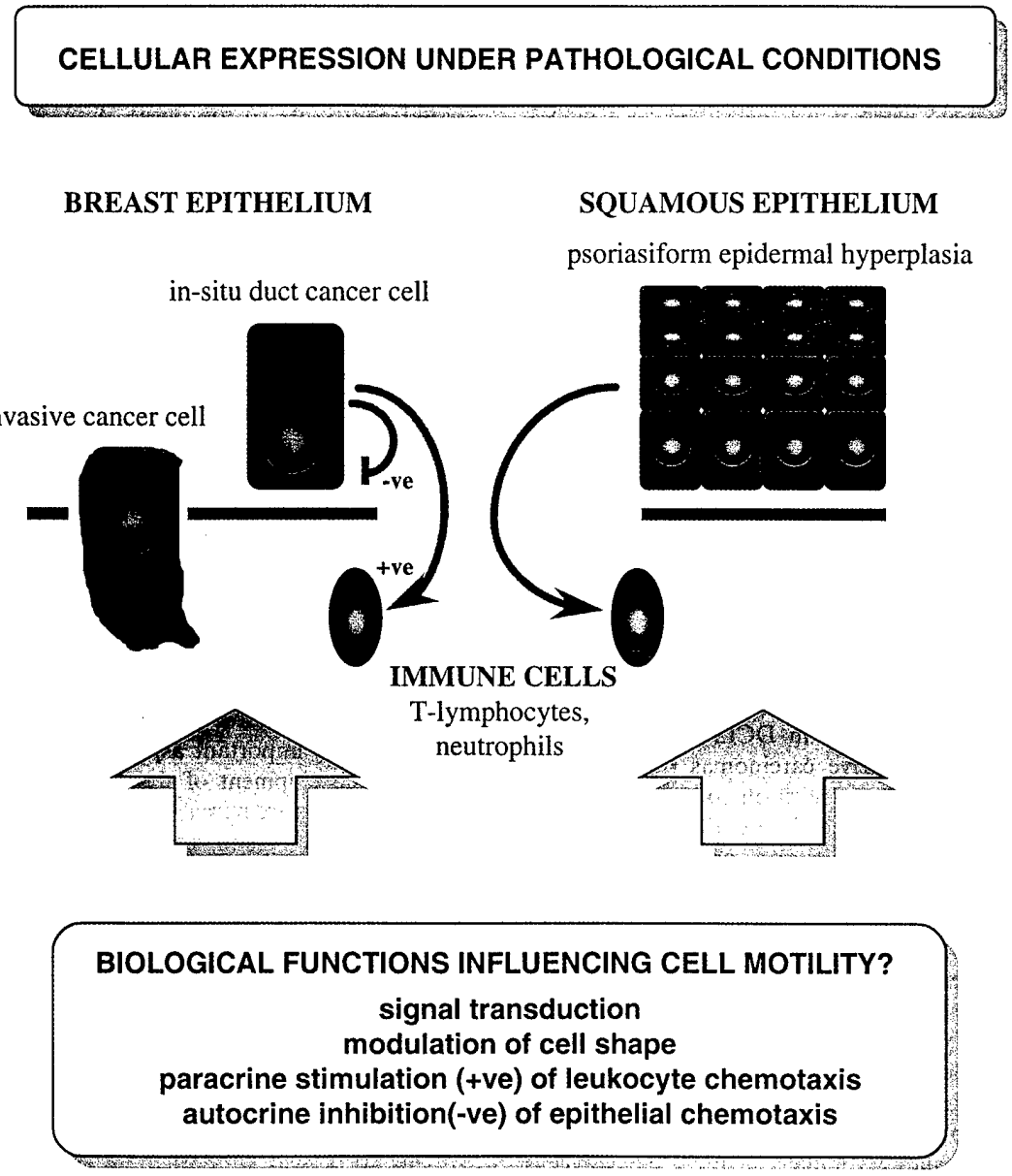

Fig. 2. A schematic representation to illustrate the possible influence of altered psoriasin expression on motility of immune and epithelial cells by intracellular actions (cell signalling and shape) and extracellular actions (chemotaxis) to mediate its role in the pathogenesis of psoriasis in skin and invasion in breast cancer.

igenesis and specifically in the process of invasion and metastasis [7]. One important component of the intracellular calcium signalling system is the S100 family of genes, which are known to be frequently expressed in breast cancer cell lines and tissues [12], but with often intriguingly different patterns of regulation that illustrate the cell type and stage-specific regulation of expression that is characteristic of the S100 family.

A possible role for Psoriasin in breast cancer first emerged when it was identified as a cDNA downregulated in a nodal metastasis relative to a primary invasive breast tumor [10]. Nevertheless, this initial observation contrasted with the fact that expression was only detectable in scattered invasive primary tumor cells by *in situ* hybridization and overall could be detected in less than 18% of primary invasive carcinomas by Northern analysis. The explanation for this paradox most probably lies in the composition of the cases studied. This has become apparent since Psoriasin was later identified as a gene highly expressed in the ductal epithelial cells of pre-invasive ductal carcinoma *in situ* (DCIS), which can be present as a significant component within invasive tumor tissue. Furthermore, study of Psoriasin mRNA expression at different stages of breast tumorigenesis has shown it to be relatively low or undetectable in normal, benign and atypical hyperplastic proliferative ductal lesions, but often highly expressed in DCIS and low or undetectable in invasive carcinomas [8]. This pattern suggests that Psoriasin overexpression is associated with altered differentiation in glandular epithelium of DCIS and may have a role in early breast tumor progression. Additional support for involvement in breast tumor progression is provided by its chromosomal location in a region of chromosome 1 that frequently ($>50\%$) shows loss of heterozygosity in invasive tumors [11].

It is therefore interesting to speculate that Psoriasin is important in the development of the invasive phenotype. This might be mediated through an indirect influence on the effector cells of the host immune response or through a more direct influence on epithelial cell motility (as illustrated in Fig. 2). While no chemotactic effect on epithelial cells has been demonstrated as yet, it is clear that the closely related S100 proteins A8 and A9 and their heterodimeric form can exert inhibitory or stimulatory chemotactic effects on different cell types under different conditions [3]. It is also known that other epithelial-derived proteins can be chemotactic for both macrophages and keratinocytes [15]. Thus, Psoriasin might exert an epithelial migration inhibitory function in *in situ* breast carcinoma cells that once lost could contribute to the onset of successful invasion.

6. In the future

It is now important to identify the receptor and/or cellular targets of a protein that is so highly expressed under certain conditions and stages of epithelial differention, in order to understand its biological function in inflammatory skin disease as well as in neoplasia. Such knowledge might on the one hand become useful in the development of new strategies to block the local epidermal inflammatory response that characterizes psoriasis, and on the other hand for the application of a clinical marker for squamous cell carcinoma of the bladder. In breast cancer, current data suggest a potential role as a clinical marker of an important aspect of cancer pathology, the development of the invasive phenotype from ductal carcinoma *in situ*. Detection of Psoriasin might distinguish *in situ* from invasive cancer in fine needle aspirates where this distinction is not always easy. This potential, as well as determination of any direct role in the process of invasion, remains to be pursued.

Acknowledgements

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APPENDIX C

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Microdissection RT-PCR Analysis of Gene Expression in Pathologically Defined Frozen Tissue Sections

Tamara Hiller, Linda Snell and Peter H. Watson

University of Manitoba, Winnipeg, MB, Canada

Molecular studies of mRNA gene expression in human solid tumors are critically dependent on the ability to apply sensitive assays to tumor tissue that is of the highest quality with respect to pathological definition and cellular preservation. In particular, the interpretation of any analysis must recognize the problems that are posed by variability in cellular composition.

Although the level of mRNA can be assessed by a variety of techniques, many such as Northern blot and RNase protection assay are not sensitive enough to allow the study of small tumor samples that are usually available for research. Alternatively, in situ hybridization allows the assessment of individual cell expression. However, sensitivity, accurate quantitation and determination of mRNA structure can sometimes be a significant limitation. The reverse transcription polymerase chain reaction (RT-PCR) assay offers a sensitive alternative that can allow accurate measurement of both structure and level of mRNA based on very small samples.

We and others have previously demonstrated the feasibility of extracting DNA from microdissected regions within archival formalin-fixed and paraffin-embedded tissue sections to assess alterations in gene structure by PCR (4,10,11). Several groups have also reported RNA extraction from paraffin sections (1,6–9). However, this can require specialized approaches to tissue fixation (6), and our experience is that this allows the amplification of only relatively stable and abundant RNA species, such as "housekeeping genes" (3,8). Alternatively, RNA can be extracted from frozen tissues; however, this has the limitation of suboptimal histological detail for the assessment of tumor parameters and precise cellular composition. In this report, we describe an approach to facilitate microdissection of small pathologically defined regions from frozen tumor sections to provide mRNA for the analysis of gene expression by RT-PCR.

Fresh tissue samples from breast cancer cases are obtained through a standardized and timed collection protocol instituted by the National Cancer

(Reprinted from BioTechniques 21:38-44, July 1996)

Institute of Canada-Manitoba Breast Tumor Bank. Portions of these tissues (typically 0.5 cm^3) are then rapidly bisected, orientated on the external and cut surfaces with different colored dyes (black india ink, alcian blue and mercurochrome) and one-half placed in 10% neutral buffered formalin, and the other is snap-frozen in liquid nitrogen within a cryovial. The fixed-tissue blocks are then processed routinely to produce matching and "mirror image" formalin-fixed, paraffin-embedded and frozen tissue blocks. Thin (5 μm) hematoxylin and eosin (H&E)-stained sections are then prepared from the paraffin blocks to allow interpretation of the detailed histology and tumor composition by light microscopy. The corresponding frozen blocks can then be sectioned in a cryostat to provide thin 5- to 20-μm sections when required, in which the distinction of tumor grade, mitotic rate, in situ tumor vs. florid ductal hyperplasia and other subtle features can be determined by direct comparison with the adjacent high-quality paraffin section.

High-quality total RNA is extractable from whole 20-μm frozen tumor sections using a small scale extraction protocol (TRI Reagent™; Molecular Research Center, Cincinnati, OH, USA) to provide an average yield of 4 $\mu g/cm^2$/20-μm tumor section (consistently optical density $[OD]_{260/280} > 1.8$ as quantitated by spectrophotometer in a 50-μL microcuvette). Although this varies with the tumor cellularity, a typical tumor section measuring 0.25 cm^2 yields 1 μg of total RNA, which is sufficient to be used as a substrate for multiple RT-PCR assays. We have used this approach to reliably amplify a range of gene products such as c-*myc*, *pS2* and *CD44*. We have also examined the effect of reuse and storage on the yield and quality of RNA that can be extracted from frozen tissue sections. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and estrogen receptor (ER) gene expression were assessed by RT-PCR using RNA extracted from frozen tissue sections, obtained from four different tumor cases. These had previously been sectioned for successful RT-PCR analysis and then re-frozen from 1½ to 2½ years previously. We

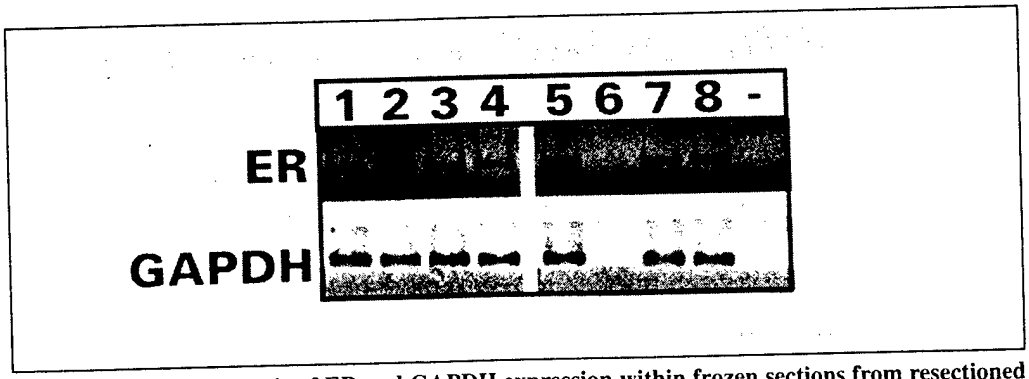

Figure 1. RT-PCR analysis of ER and GAPDH expression within frozen sections from resectioned tumor blocks. RNA was extracted from frozen sections obtained from tumor blocks that had been sectioned and successfully used for RNA extraction and RT-PCR up to 2½ years previously. In all four tumors, the expression of ER and GAPDH can be readily detected in the reused blocks (lanes 1–4), and this is similar to the expression seen in the corresponding samples of RNA stored at -70°C since the original extraction (lanes 5–8). Note that the integrity of one stored RNA sample (lane 6) has been lost.

have found no significant loss of RT-PCR signal in RNA extracted from previously sectioned frozen blocks by comparison with the original RNA extracted (Figure 1) when the tissue blocks are stored at -70°C and carefully handled during processing. By comparison, and as illustrated by the tumor in Figure 1, lane 6, degradation of stored RNA samples can occur through incomplete removal of endogenous tissue RNases or contamination by exogenous RNases introduced during RNA extraction or tube storage (5).

To assess gene expression in a defined region within a tissue section, several 20-µm frozen sections may be cut from the frozen tumor block (in a Leica cryostat at -30°C; Leica, Willowdale, Ontario, Canada) and each then mounted onto a glass slide that has been previously coated with 2% agarose (Boehringer Mannheim, Laval, PQ, Canada) for microdissection using a modification of a method previously described for assessment of enzyme activity (2). Frozen sections directly mounted onto glass slides dry out rapidly during dissection and are difficult to dissect. Slides coated by 2% agarose, to a depth of 1 mm, can be prepared by pouring molten agarose in autoclaved ddH_2O onto slides. These are then stored at 4°C for up to 1 h before use to prevent dessication. Mounted sections are immersed in a Harris's hema-

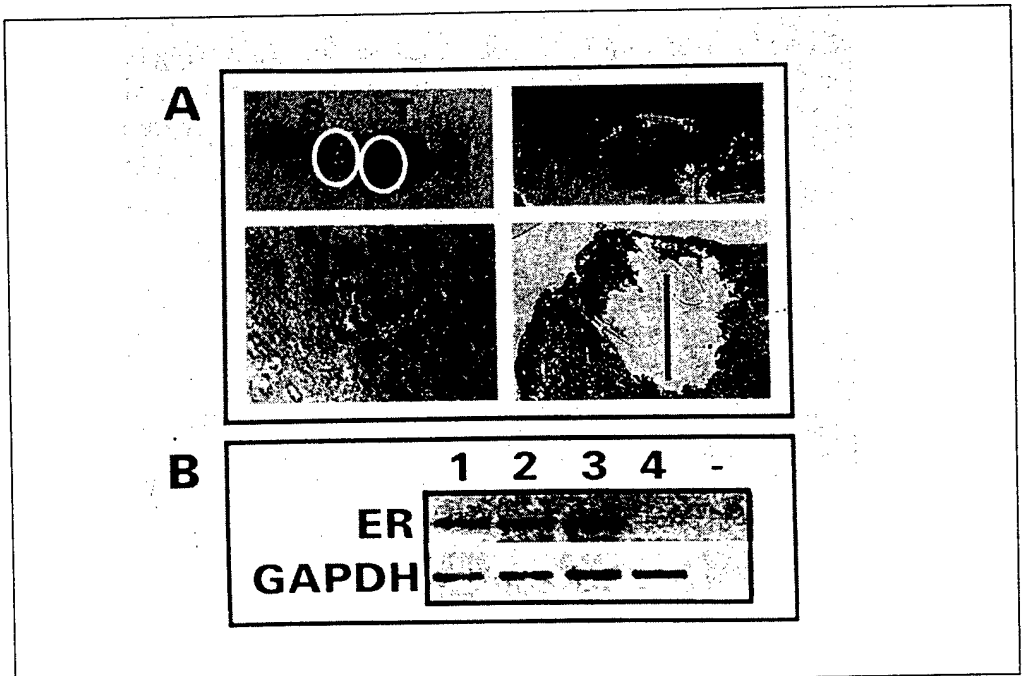

Figure 2. Microdissection/RT-PCR analysis of ER and GAPDH expression within a breast tumor section. Upper panel (A) shows original H&E-stained frozen section from a breast tumor with separate regions consisting of invasive tumor and stroma (top left); the adjacent serial section mounted on agarose after microdissection (top right); the detail of the histology of the lymphocyte-rich stroma and tumor (bottom left) and the microdissected tumor region (bottom right, scale bar = 0.2 cm). Lower panel (B) shows the results of RT-PCR analysis of ER and GAPDH expression from the entire frozen tumor section (lane 1), the entire H&E-stained frozen tumor section (lane 2), the microdissected region of invasive tumor (lane 3), the microdissected region of stroma (lane 4) and the RT-PCR negative control (RNA-).

toxylin solution (Mallinckrodt, Winnipeg, MB, Canada) for 2 s at room temperature, rinsed in water for 10 s, immersed into Eosin Y in 95% ethanol (Mallinckrodt) for 2 s, rinsed again in water for 10 s and then placed under a dissection microscope (wild M3C; Leica). Sections can then be oriented and the histological details confirmed with reference to a paraffin H&E-stained section from the matching paraffin block. Using a scalpel blade and fine needle, specific tumor components within histologically defined areas less than 1–2 mm^2 can be rapidly microdissected within 2 min under a 20× objective at room temperature and placed into a precooled microcentrifuge tube on ice. After a brief centrifugation to pellet the dissected material, 10 µL ddH_2O are added, and the material is snap-frozen by immersion in liquid nitrogen to disrupt tissue architecture. RNA can then be extracted and used as a substrate for RT-PCR assay. For a typical 20-mm breast tumor section, which may contain 10^4 cells within a 2-mm region, we obtain yields of between 0.5–1 µg RNA from 4 microdissected serial sections. The brief H&E stain provides essential cellular discrimination without significantly affecting the ability to perform successful RT-PCR.

To demonstrate the feasibility of this approach, a microdissection experiment performed on tissue from an ER- positive carcinoma is shown in Figure 2. The mRNA expression of the GAPDH "housekeeping" gene is detected in the entire unstained frozen tumor section, in the section following brief H&E staining and in both microdissected tumor and stroma regions. Similarly, expression of ER is seen in the entire tumor section and in the region of invasive carcinoma, but as expected, it is absent in the immediately adjacent lymphocyte-rich stroma.

Following microdissection, 100 ng total RNA from each component were reverse-transcribed in a volume of 20 µL of RT mixture (1× RT buffer and 200 U Moloney murine leukemia virus [MMLV] RTase (Life Technologies, Burlington, ON, Canada); 0.5 mM each dGTP, dATP, dTTP, dCTP; 1 mM bovine serum albumin [BSA]; 0.01 M dithiothreitol [DTT]; 1.25 mM oligo(dT) primer; 5% dimethyl sulfoxide [DMSO]) and incubated for 60 min at 37°C. PCR amplification of GAPDH and ER cDNA was then performed in a Model PTC-100™ thermal cycler (MJ Research, Watertown, MA, USA). Each PCR was performed in a 50-µL volume utilizing 2 µL of the completed RT reaction containing cDNA; 1× PCR buffer; 2 µM $MgCl_2$; 1.1 U *Taq* DNA polymerase (Promega, Unionville, ON, Canada); 200 mM each dGTP, dATP, dTTP and dCTP; and 0.5 mM PCR primers. The PCR protocol consisted of 5 min at 94°C; then 40 cycles of 45 s at 93°C, 45 s at 56°C and 90 s at 75°C; followed by 7 min at 72°C. After thermal cycling was completed, 1.5 µL of gel loading buffer were added to 15 µL of the PCR, and samples were electrophoresed on a 2% agarose gel. PCR products were visualized by subsequent ethidium bromide staining and photography under UV light. The primer sequences used were as follows; $GAPDH^{943}$ 5′ ACCCACTCCTCCACCTTTG 3′, $GAPDH^{1102}$ 5′ CTCTTGTGCTCTTGCTGGG 3′, ER^{675} 5′ TGC-

CCTACTACCTGGAGAA 3′, ER 5′ TGGTAGCCTGAAGCATAGTC 3′.

In conclusion, we have described an approach involving a specific protocol for tissue processing that allows for the extraction of mRNA from single histologically defined tumor sections. We have also shown that it is feasible to microdissect small areas from within H&E-stained frozen tumor sections and to extract RNA that is suitable for RT-PCR analysis of specific components within tumors.

ACKNOWLEDGMENTS

This work was supported in part by grants from the Canadian Breast Cancer Research Initiative, the National Cancer Institute of Canada and the US Army Research & Development Command. The material was provided by the NCIC-Manitoba Breast Tumor Bank. P.H.W. is Medical Research Council of Canada Clinician-Scientist.

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Update to:

Microdissection RT-PCR Analysis of Gene Expression in Pathologically Defined Frozen Tissue Sections

P.H. Watson, T. Hiller, L. Snell, K. Hole, R. Singh, L.C. Murphy, E.T. Leygue, H. Dotzlaw, A. Kossakowska[1] and A. Kulakowski[2]

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We have used this approach to pursue the specific goal of identification of genes that influence growth and invasiveness in human breast cancer (1,3). Several indirect approaches to the latter problem have been taken by others, including either assessment of known genes that may be important for invasion on the basis of their discovery and their roles in other experimental systems, or the search for DNA loci with genetic damage (loss of heterozygosity) that might indicate alteration of expression of genes involved in invasion. However, through microdissection RT-PCR of pathologically defined in situ and invasive elements within frozen breast tumor sections and examination of RNA for alterations of gene expression, we have been able to take a more direct approach to this problem. In pursuit of this direction we have made the following progress.

To improve our ability to interpret alterations in levels of gene expression in vivo, we have established a tissue collection protocol and studied the effect of tumor collection time on RNA expression in breast tumor specimens. We have used competitive RT-PCR assays to measure the levels of expression of both relatively stable (estrogen receptor [ER]) and unstable (c-*myc*) RNAs expressed in breast tumors collected and frozen over different times. We have found that the level of c-*myc* RNA declines more rapidly than ER, even in tissues collected and maintained on ice, with mean c-*myc* levels falling to 80% of initial level at 24 h as compared to mean ER levels of 94% of initial levels. Therefore, although differences in RNA levels may not be significant with shorter collection periods, our results show that standardization of the method of tissue collection is important as time to collection can influence mRNA levels of some genes which decay faster than others.

We have also examined the ability to apply our approach to tissue processing, microdissection and RT-PCR to tissues obtained from distant sources by examining breast tumor material transported from Poland, in collaboration with the Warsaw Cancer Institute. Morphological tissue quality assessed on a

three-point scale in paraffin block sections is very comparable to local tissues, and GAPDH and ER are also readily amplified from RNA extracted from the parallel frozen tissue sections (5).

In conjunction with the microdissection RT-PCR approach we have developed and tested a novel subtraction hybridization technique (2), modified from previous methods so that it can be applied to the small amounts of mRNA available from microdissected breast lesions.

We have then successfully applied this combined microdissection and subtraction hybridization approach to directly compare gene expression between pre-invasive in situ (DCIS) and adjacent early invasive tumor cell populations within the same tumor specimen (3,4). This has led to the identification of psoriasin (3), a member of the S100 calcium binding protein family, as a candidate "invasion suppressor" gene. We have gone on to show that psoriasin is differentially expressed in DCIS vs. invasive tumor components by in situ hybridization and RT-PCR in other cases as well as in the sentinel tumor. Our overall tumor tissue banking approach (5) makes the latter feasible as these expression studies can be applied to resectioned and dissected material from the same or parallel tissue block that was originally microdissected.

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APPENDIX D

Manuscript in preparation.

Psoriasin(S100A7) expression in human breast carcinoma.

Al-Haddad S, Zi Yuan, Huang A., Leygue E., Hiller T., Snell L., Murphy L., Watson P.

Affiliations of authors: Department of Pathology (S.H., L. S.,T. H-H., A. H., P. H. W.) and Department of Biochemistry and Molecular Biology (E. L., L. C. M.), University of Manitoba, Faculty of Medicine, Winnipeg, Manitoba, Canada, R3E OW3.

Running title: Psoriasin expression in breast cancer.

Key words: Psoriasin, S100A7, breast cancer, tumor progression.

Footnotes:

1 This work was supported by grants from the U.S. Army Medical Research and Materiel Command (USAMRMC) and the Medical Research Council of Canada (MRC). The Manitoba Breast Tumor Bank is supported by funding from the National Cancer Institute of Canada (NCIC). P. H. W. is an MRC Clinician-Scientist, L. C. M. is an MRC Scientist, E. L. is a recipient of a USAMRMC Postdoctoral Fellowship. T. H-H is a recipient of an MRC studentship award.

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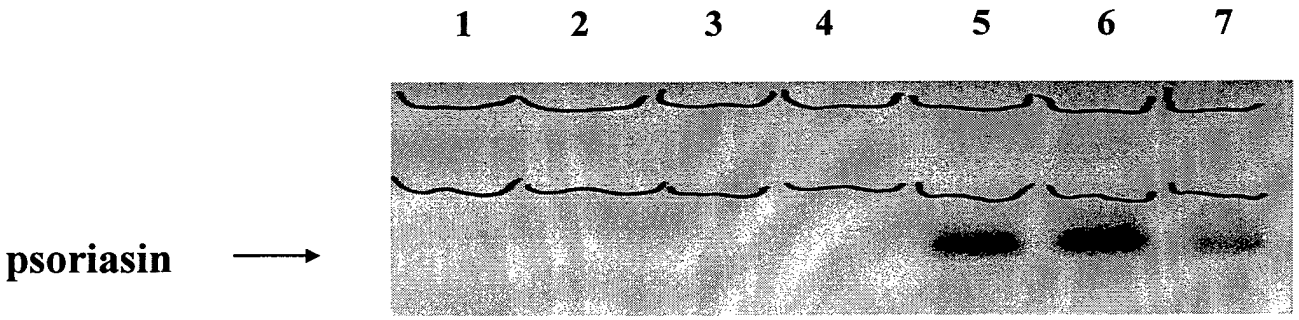
3 The abbreviations used are: RT-PCR, reverse transcription-polymerase chain reaction; H&E, Hematoxylin/Eosin; ER, estrogen receptor; PR, progesterone receptor; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

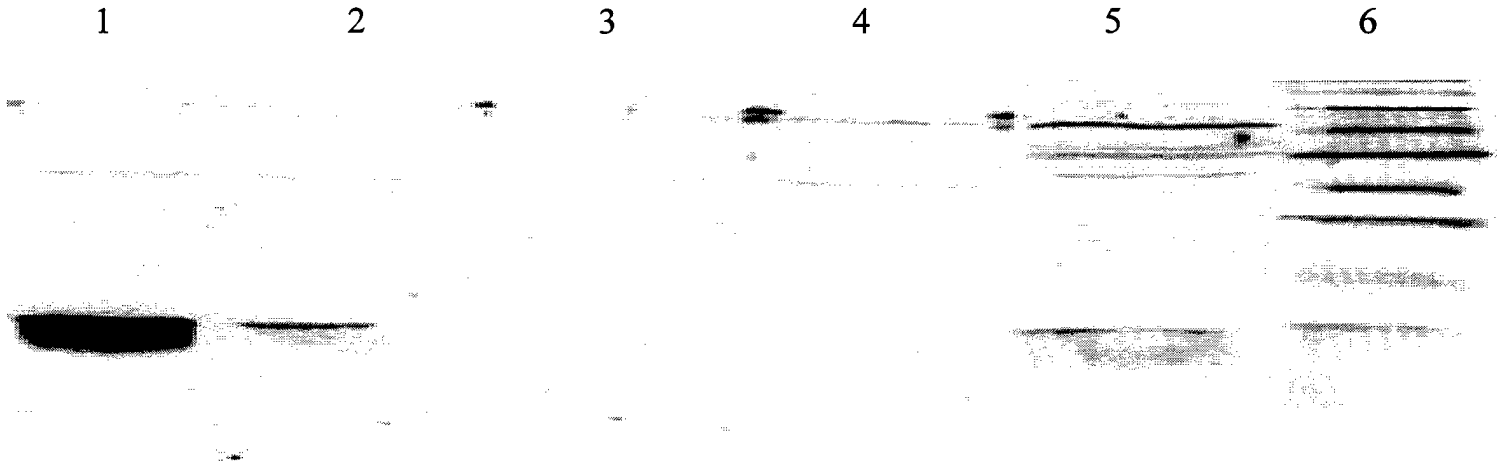
EXPRESSION OF PSORIASIN mRNA IN STABLE TRANSFECTED MDA-MB-231 CELLS



- Northern blot of multiple MDA-MB-231 cell clones.
- Lanes 1 & 2, transfected with vector alone, lanes 3 & 4 transfected with CMV-Psoriasin cDNA in the reverse orientation, lanes 5 to 7 transfected with CMV-psoriasin cDNA.

Fig 1

EXPRESSION OF PSORIASIN PROTEIN IN STABLE TRANSFECTED MDA-MB-231 CELLS

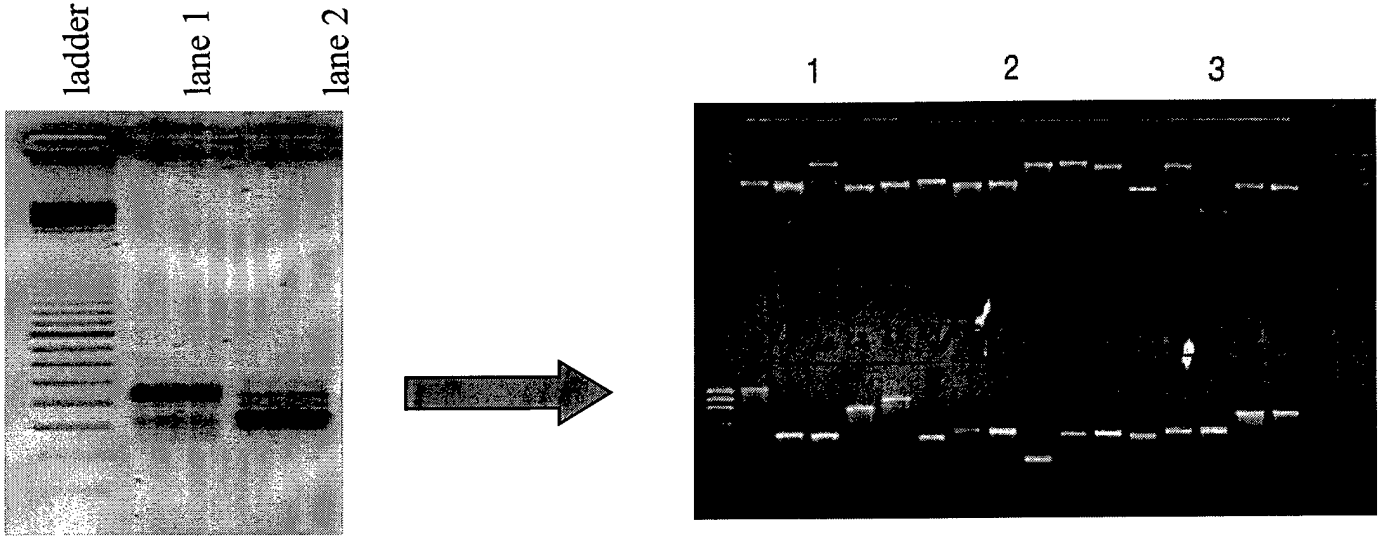


Western blot performed using anti-psoriasin IgG ab kindly provided by Dr Celis, Denmark.

- lane 1 - Psoriasin transfected MDA-MB-231 clone showing high levels of expression
- lane 2 - Psoriasin transfected MDA-MB-231 clone showing low levels of expression
- lane 3 - Vector alone transfected MDA-MB-231 clone
- lane 4 - MDA-MB-231 cells (negative control)
- lane 5 - MCF7 cells treated with estradiol (48 hrs)
- lane 6 - ladder

Fig 2

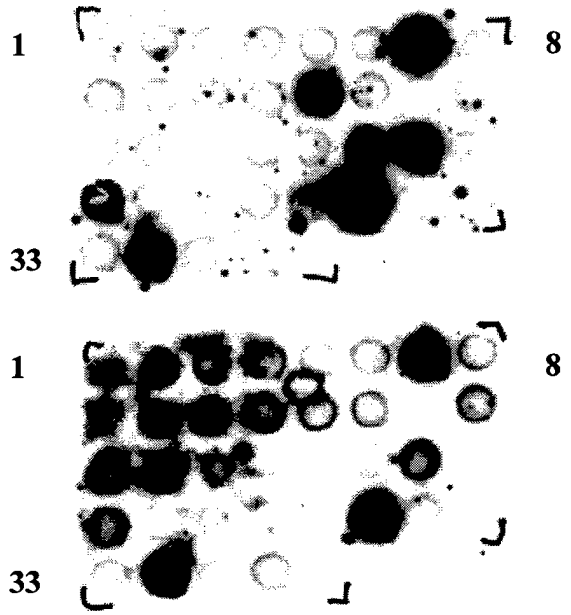
Representational Difference Analysis assay performed on psoriasin transfected MDA-MB-231 cells vs. non-transfected parental cells



- LEFT. Agarose gel stained with ethidium bromide showing multiple bands representing potential differentially expressed cDNA's overexpressed in transfected and parental cells respectively. Cell lines used were CLC6FA1 psoriasin transfected and CLCVA1 vector alone. Lane 1: CLC6FA1 as tester against CLCVA1 as driver. Lane 2: CLCVA1 as tester against CLC6FA1 as driver
- RIGHT. Agarose gel stained with ethidium bromide showing multiple cDNA's amplified from plasmid insert clones derived 6 of the major bands in samples shown on the left, after separation, isolation by dissection, and reamplification from an acrylamide gel. Variation in band size reflects incomplete separation and purification and the presence of additional cDNA's adjacent to the band of interest, and cloning contamination. Those bands which show consistent size and replication in multiple clones are currently undergoing sequencing and further analysis.

Fig 3

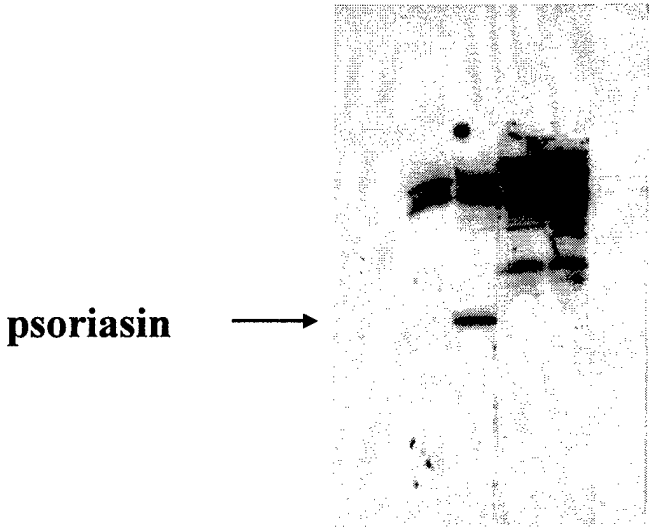
Analysis of cDNA clones from Representational Difference Analysis assay performed on psoriasin transfected MDA-MB-231 cells vs. non-transfected parental cells



- Equal amounts of 36 cDNAs obtained after RDA and corresponding to genes potentially overexpressed in psoriasin transfected MDA-MB-231 cells relative to parental MDA-MB-231 cells has been blotted on two different nitrocellulose membranes (upper and lower panels). Each panel has been probed with cDNA labelled by nick translation from the initial representation step from each cell line (vector alone, top panel and psoriasin transfected, lower panel). Some cDNAs (#'s 7, 30, 34) are apparently expressed at the same level in both cell populations, however, some are differentially expressed. cDNA #13 is more expressed in control cells, whereas cDNAs #'s 1-4, 9-1 and 18 are likely to be overexpressed in psoriasin transfected cells.

Fig 4

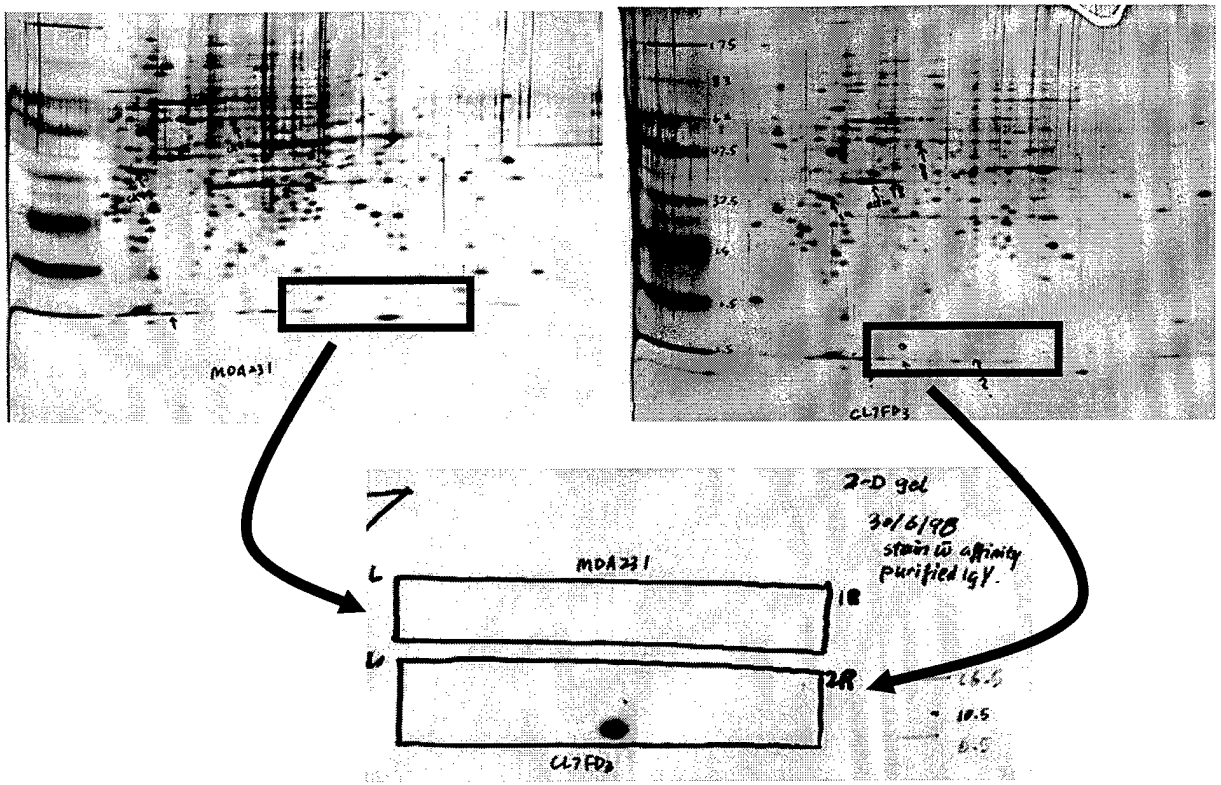
Western blot performed on wild type and transfected MDA-231 cells using either Ig-Y alone (left lanes 1&2) or Ig-Y + psoriasin peptide used for immunization (right lanes 3&4) showing specificity of anti-psoriasin antibody



- lane 1 & 3 - MDA-MB-231 parental cells (negative control)
- lane 2 & 4 - MDA-MB-231 psoriasin transfected cells

Fig 5

WESTERN BLOT2D-SDS PAGE ANALYSIS OF ANTI-PSORIASIN IG-Y ANTIOBODY



- Upper panel shows 2-D Gel analysis of MDA-231 wild type (left) and transfected (right) cells.
- Lower panel shows Western blot applied to selected regions of each 2-D gel and shows specific psoriasin staining for one differentially expressed protein that lies in the region expected for psorasin(molecular weight and PI) . (Differences in gel conditions, spot intensities, and cell variation inherent in cell cloning, and perhaps the effect of overexpression of psoriasin, provide several other differences between these cells).

Fig 6

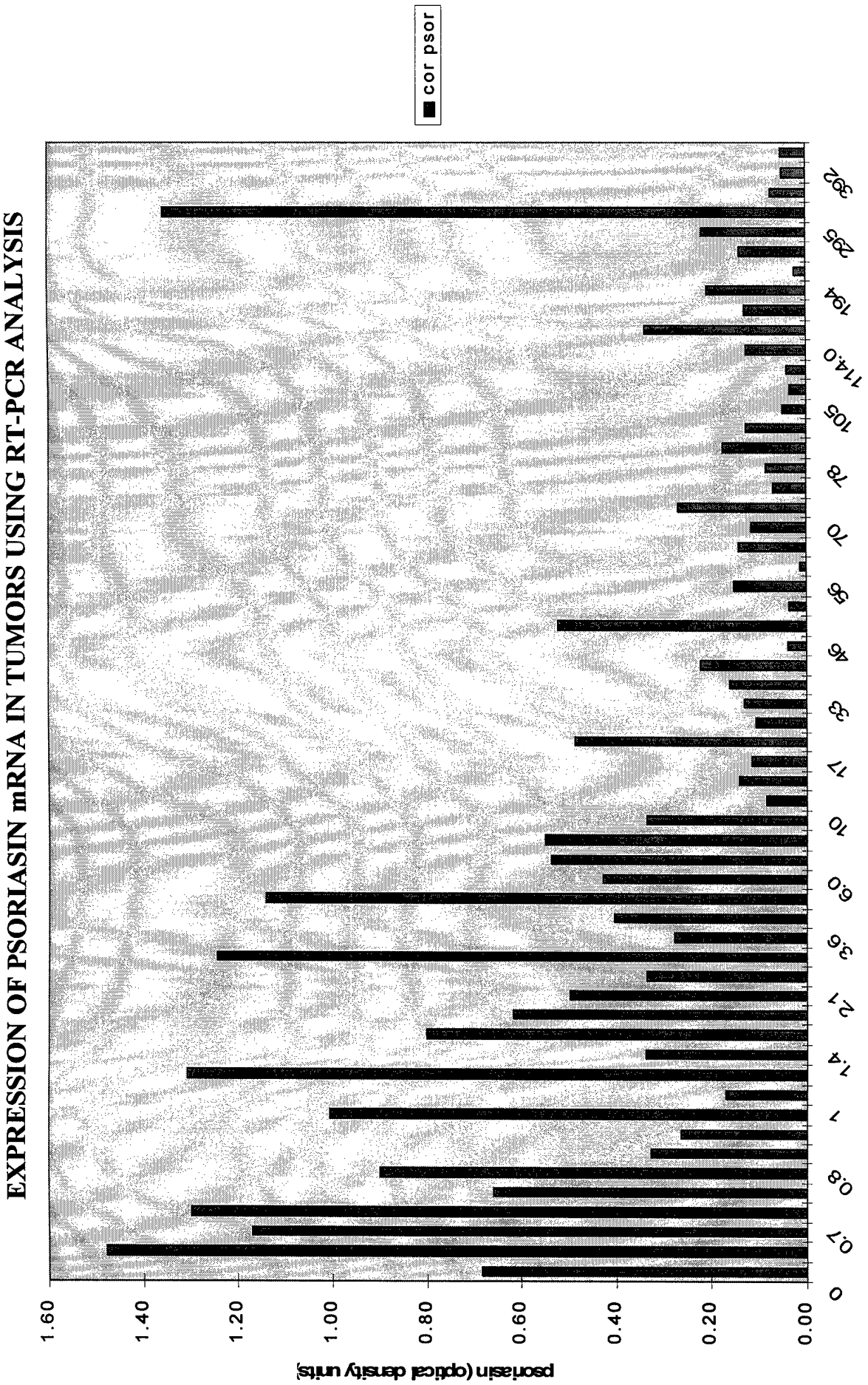
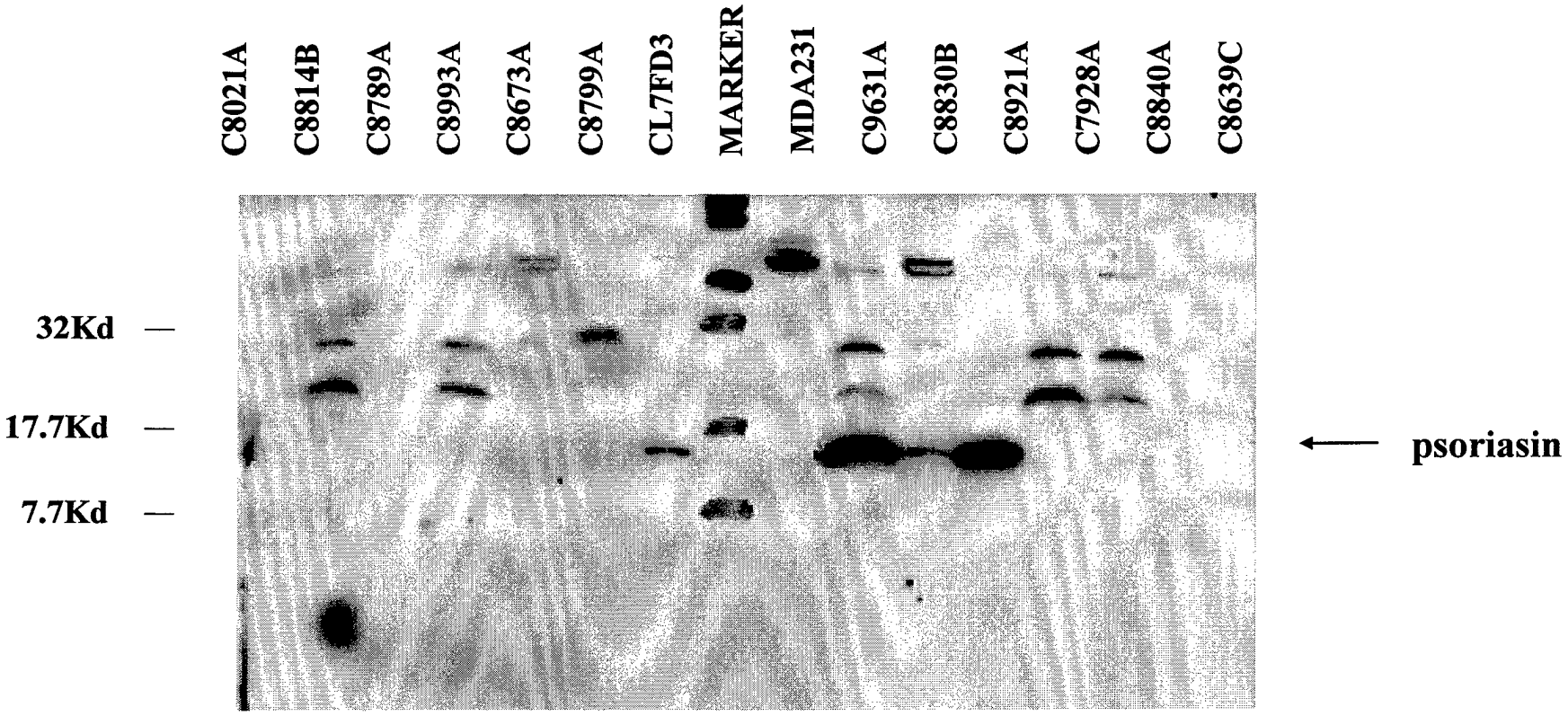

EXPRESSION OF PSORIASIN mRNA IN TUMORS USING RT-PCR ANALYSIS
cor psor
psoriasin (optical density units)
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0
0.7
0.8
1
1.4
2.1
3.6
6.0
10
17
33
46
56
70
78
105
114.0
194
295
392
er levels (fm mols/mg)

Fig 7

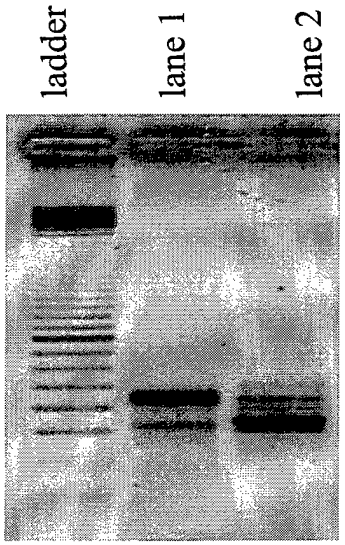
EXPRESSION OF PSORIASIN IN TUMORS USING WESTERN BLOT ANALYSIS



- Western blot performed with anti-psoriasin Ig Y.
- Lanes 1-6 and 10-15 correspond to Tumor Bank tumor cases
- lanes 7 & 9 correspond to MDA-231 psoriasin transfected and wild type cells respectively.
- lanes 8 corresponds to Molecular Weight Size marker

Fig 8

Representational Difference Analysis assay performed on psoriasin transfected MDA-MB-231 cells vs. non-transfected parental cells



- Agarose gel stained with ethidium bromide showing multiple bands representing potential differentially expressed cDNA's overexpressed in transfected and parental cells respectively. Cell lines used were CLC6FA1 psoriasin transfected and CLCVA1 vector alone. Lane 1: CLC6FA1 as tester against CLCVA1 as driver. Lane 2: CLCVA1 as tester against CLC6FA1 as driver

Fig 3

APPENDIX E

[CANCER RESEARCH 58, 1348–1352, April 1, 1998]

Advances in Brief

Expression of Lumican in Human Breast Carcinoma[1]

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Abstract

Lumican mRNA has been identified as being differentially expressed between different regions of the same human breast tumor. *In situ* hybridization study of 26 independent breast tumors confirmed the presence of lumican mRNA in fibroblast-like cells within stroma and showed a significant increase of its expression in tumor compared to adjacent normal stroma ($P < 0.001$). Higher lumican expression was associated with higher tumor grade, lower estrogen receptor levels in the tumor, and younger age of the patients ($P < 0.05$). Reverse transcription-PCR analysis of total RNA extracted from 19 independent breast tissues exhibiting lesions that are thought to parallel tumor progression also suggests that this proteoglycan is differentially expressed during tumor progression.

Introduction

The molecular mechanisms underlying the transition of normal breast tissue to invasive breast tumor *in vivo* are still poorly understood. It is believed that sequential genetic alterations that contribute to the development of phenotypic changes, such as deregulated proliferation and the cytological appearance of malignancy, accumulate in epithelial cells. Paralleling these changes in epithelial cells, changes in expression of genes within fibroblasts surrounding tumor lesions have been described (1–4) and are believed to signal complex interactions between transformed epithelial cells and adjacent host stromal cells that also contribute to the progression of the tumor (3). Knowledge of the mechanisms underlying these interactions and identification of genes differentially expressed during tumor progression would allow a better understanding of tumorigenesis and might provide targets for new therapies (5). To address this issue, we have undertaken a study to identify genes differentially expressed during tumorigenesis (6). Using a recently described subtractive hybridization technique (7), we have here identified lumican mRNA as being differentially expressed between two regions of the same tumor biopsy sample. The expression of lumican has then been investigated by *in situ* hybridization and RT-PCR[3] in independent breast tissue specimens.

Materials and Methods

Human Breast Tissues and Cell Lines. All breast tumor cases used for this study were selected from the National Cancer Institute of Canada-Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). As described previously (8), tissues from all cases are rapidly collected and processed to create formalin-fixed and paraffin-embedded tissue blocks and mirror-image frozen tissue blocks. The precise histological detail, interpreted in H&E-stained sections from the former block, was used as a guide to microdissection of the frozen block. Four groups of tissues or cell lines were used in this study, as detailed below.

For initial microdissection, a single tumor case with extensive *in situ* and limited (<25%) invasive components was selected from the Manitoba Breast Tumor Bank. Both components were present within a single frozen tissue block. For the *in situ* hybridization studies, 26 tumors were selected to allow comparison of normal ducts/lobules and *in situ* and invasive tumor elements within a single section in each case. For subsequent statistical analyses, cases were divided into groups of lower-grade [Nottingham (9) scores of 4–6; $n = 12$] and higher-grade (Nottingham scores of 7–9; $n = 14$) tumors; younger ($n = 14$) and older ($n = 12$) than the mean patient age (54 years old); higher ER (ER > 20 fmol/mg protein; $n = 10$) and lower ER (ER < 20 fmol/mg protein; $n = 16$) status tumors, as determined by ligand-binding assay; higher PR (PR > 15 fmol/mg protein; $n = 14$) and lower PR (PR < 15 fmol/mg protein; $n = 12$) status, as determined by ligand-binding assay; or axillary node-positive ($n = 15$) and -negative ($n = 11$) tumors.

For RT-PCR analysis of lumican expression in breast lesions that are thought to parallel the development of breast cancer, a second panel of 19 breast tissue samples, corresponding to 19 different patients, was selected. This panel consisted of five cases with normal breast tissue, four cases with proliferative disease without atypia, five cases with ductal carcinoma *in situ*, and five cases of invasive ductal carcinoma. In all cases, the histology of the frozen tissue block that was used was determined by direct comparison with an adjacent H&E-stained section from a mirror-image paraffin block.

For study of cell lines, both ER-positive and -negative breast cancer cells (T47-D, T47-D5, MCF-7, BT20, MDA-MB-231, and MDA-MB-468) were grown as described previously (10).

Microdissection, RNA Extraction, and Subtractive Hybridization Analysis. A single tumor case was microdissected as described previously (8), and RNA was extracted from histologically defined regions of *in situ* and invasive tumor using a small-scale RNA extraction protocol (Tri-reagent; MRCI, Cincinnati, OH). The subtractive hybridization was performed as described previously, using the *in situ* and invasive component as the tester and the driver, respectively (7). Total RNA was similarly extracted from frozen sections of other breast samples or cell lines.

***In Situ* Hybridization.** Paraffin-embedded 5-μm breast tumor sections corresponding to tumor specimens were analyzed by *in situ* hybridization according to a previously described protocol (6). The plasmid Lumi-398, which consisted of PCRII plasmid (Invitrogen, San Diego, CA) containing a 398-bp insert of lumican cDNA between bases 1332 and 1729 (11), was used as a template to generate [^{35}S]UPT-labeled sense and antisense riboprobes using Riboprobe Systems (Promega, Madison, WI), according to the manufacturer's instructions. Sections were then developed and counterstained with H&E after 7–21 days. Levels of lumican expression were assessed in normal and tumor regions within each section by brightfield microscopic examination at low-power magnification and with reference to a positive control tumor section and a control normal tissue section. This was done by scoring the estimated average signal intensity (on a scale of 0–3) and the proportion of stromal cells showing a positive signal (0, none; 0.1, less than 1/10; 0.5, less than 1/2; 1.0, greater than 1/2). The intensity and proportion scores were then multiplied to give an overall score. Regions with a score lower than 1.0 were deemed negative or weakly positive.

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[1] This work was supported by grants from the Canadian Breast Cancer Research Initiative (CBCRI) and the United States Army Medical Research and Materiel Command (USAMRMC). The Manitoba Breast Tumor Bank is supported by funding from the National Cancer Institute of Canada (NCIC). P. H. W. is a Medical Research Council of Canada (MRC) Clinician Scientist, and L. C. M. is a MRC Scientist. E. L. is a recipient of a USAMRMC Postdoctoral Fellowship.

[2] To whom requests for reprints should be addressed. Phone: (204) 789-3812; Fax: (204) 789-3900; E-mail: eleygue@cc.umanitoba.ca.

[3] The abbreviations used are: RT-PCR, reverse transcription-PCR; ER, estrogen receptor; PR, progesterone receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

SDS-PAGE and Immunoblotting. Total proteins were extracted from frozen tissue sections corresponding to three tumors expressing lumican mRNA (as shown by *in situ* hybridization) using 4 M guanidinium chloride in the presence of proteinase inhibitors, as described previously (11). Proteins present in the extracts were analyzed by SDS-PAGE and immunoblotting, using antipeptide antibodies specific for the COOH-terminal regions of lumican (11, 12). Prior to analysis, extracts were dialyzed into 10 mM sodium acetate and treated with chondroitinase ABC. In some cases, the samples were also treated with keratanase II or endo-β-galactosidase, which are capable of degrading sulfated and nonsulfated polylactosamine chains, respectively.

RT-PCR Analysis. One μg of total RNA was reverse-transcribed in a final volume of 15 μl, and 1 μl of the reaction mixture was subsequently amplified by PCR, as described previously (6). Primers used corresponded to lumican (sense, 5′-TAAACCACAACAACCTGACA-3′, located in lumican sequence between bases 448 and 467; and antisense 5′-AGAAAAACATAACCATAAAA-3′, located in lumican sequence between bases 1118 and 1138; Ref. 11) and to the ubiquitously expressed *GAPDH* gene (sense, 5′-ACCCACTCCTCCACCTTTG-3′; and antisense, 5′-CTCTTGTGCTCTTGCTGGG-3′). To amplify cDNA corresponding to lumican, 30 cycles (1 min at 94°C, 1 min at 52°C, and 2 min at 72°C) of PCR were used. For amplification of *GAPDH* cDNA, PCR consisted of 30 cycles (30 s at 94°C, 30 s at 52°C, and 30 s at 72°C). Ten μl of lumican PCR and *GAPDH* PCR were mixed before migration on 2% agarose gels and staining with ethidium bromide (15 μg/ml). Identity of the 691-bp-long fragment corresponding to lumican was confirmed after subcloning and sequencing.

Results

Identification of Lumican mRNA in Breast Cancer. To identify genes differentially expressed during tumor progression, a "microdissection case" containing high-grade lobular carcinoma *in situ* associated with invasive lobular carcinoma was selected. Two regions, one rich in *in situ* and the other rich in invasive components, were microdissected, and corresponding total RNA was extracted to provide the substrate for a recently described subtractive hybridization technique (7). Upon completion of subtractive hybridization, a 398-bp-long fragment was isolated as corresponding to a gene being overexpressed in the *in situ* compartment of this single microdissection case (data not shown). Sequencing of this fragment identified nucleotides 1332–1729 of the sequence encoding the core protein of the keratan sulfate proteoglycan lumican (11).

***In Situ* Hybridization Analysis of the Pattern of Lumican mRNA Expression within Tumors and Adjacent Normal Tissue.** To establish the cellular origin of expression and to examine the distribution of lumican expression between different tumor components in other tumors, 26 invasive tumors were studied by *in situ* hybridization (Fig. 1). For each case, an adjacent H&E-stained section was used to facilitate the pathophysiological interpretation of the frozen section (Fig. 1*A*). In almost all cases, a similar pattern was evident, with prominent mRNA expression, detected using an antisense probe, in stromal fibroblast like cells within the tumor and immediately adjacent to *in situ* and invasive tumor cells (Fig. 1*B*). No signal was observed in any case using a lumican sense probe (Fig. 1*C*). In 24 of these cases, regions of normal tissue, *in situ* tumor, and invasive tumor were present within the single section studied, therefore allowing comparison of lumican expression within the stromal elements associated with these epithelial components. Expression of lumican was evaluated within normal and tumor compartments using a semiquantitative approach as detailed in "Materials and Methods" (Table 1). Lumican was found to be expressed at very low levels (<1) within the collagenous stromal tissues associated with normal ducts and lobules in all but 1 of the 24 cases, whereas expression was evident at higher levels (≥ 1) in stromal fibroblast-like cells within the collagenous stroma of 23 of 26 invasive tumors ($P < 0.001$; Fisher's exact test). Furthermore, a marked difference between stromal expression within adjacent matched regions of normal and invasive tumor (*i.e.*, difference between tumor and normal scores higher than 1) was present in 16 of 24 cases in which this could be directly compared (see Fig. 1*D*). In 16 of 23 cases in which lumican expression was high in the invasive tumor, the ductal or lobular carcinoma *in situ* components of the tumor were associated with equivalent or higher levels of lumican expression in the immediately adjacent periductal or perilobular stroma (see Fig. 1, *B* and *D*).

The relationship between lumican expression and prognostic factors was conducted by Fisher's exact test analysis. For this purpose, tumors were divided into two subgroups with high (>1; $n = 17$) or low (≤ 1; $n = 9$) levels of lumican expression and with different tumor characteristics (high or low tumor grade, younger and older patients, high or low ER status, high or low PR status, and presence or absence

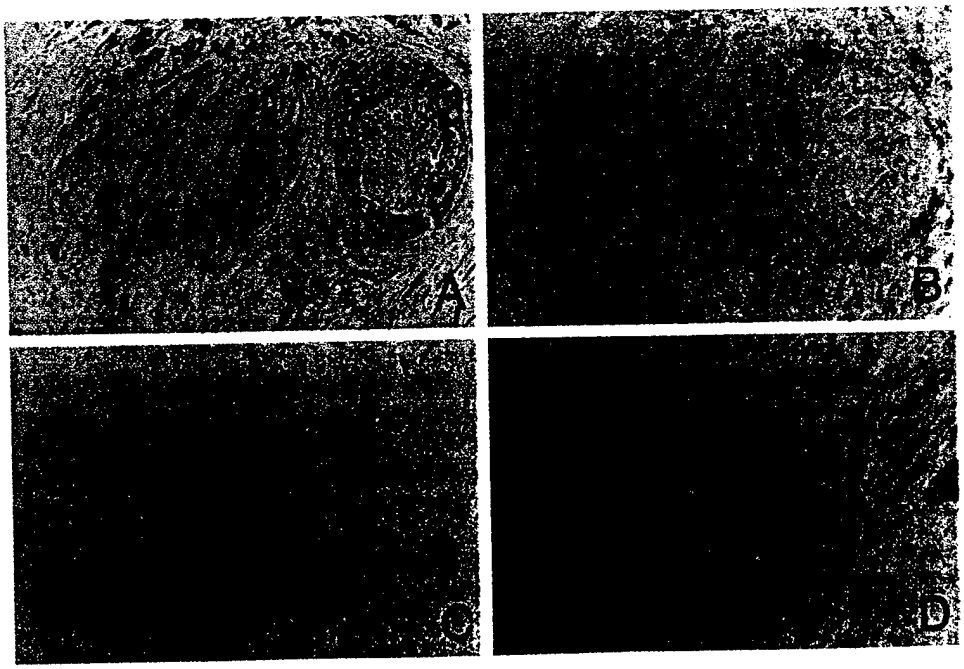

Fig. 1. Expression of lumican mRNA in breast tumors studied by *in situ* hybridization. Consecutive sections from a single breast tumor show H&E-stained paraffin section with collagenous stromal reaction surrounding ductal carcinoma *in situ* (*right*) and invasive (*left*) components (*A*), lumican expression in stroma detected using an antisense probe (*B*), and lumican sense probe (*C*). *D*, lumican expression detected within a different tumor, illustrating a gradient in the level of expression between regions of *in situ* (*top left*) and invasive ductal carcinoma (*middle*) and normal tissue (*right*). Original magnifications, ×100 (*A–C*) and ×25 (*D*).

Table 1 *Clinical and pathological features of breast tumors studied for lumican mRNA expression by* in situ *hybridization*[a]

No.	Lumican score		Tumor characteristic				
	Normal	Tumor	Nottingham grade	Age (yr)	ER status	PR status	NS
1	0.1 (0.1 × 1.0)	0.1 (0.1 × 1.0)	5	48	−	−	+
2	0.0 (0.0 × 0.1)	0.2 (0.1 × 2.0)	6	56	+	−	+
3	0.1 (0.1 × 1.0)	0.3 (0.1 × 3.0)	7	80	−	−	+
4	0.0 (0.0 × 0.0)	1.0 (1.0 × 1.0)	5	66	+	−	+
5	0.5 (0.5 × 1.0)	1.0 (0.5 × 2.0)	5	71	+	+	−
6	0.0 (0.0 × 0.0)	1.0 (0.5 × 2.0)	6	61	+	+	−
7	0.1 (0.1 × 1.0)	1.0 (0.5 × 2.0)	6	58	+	+	+
8	0.1 (0.1 × 1.0)	1.0 (0.5 × 2.0)	9	46	−	−	+
9	0.1 (0.1 × 1.0)	1.0 (0.5 × 2.0)	5	75	+	+	−
10	0.0 (0.0 × 0.0)	1.5 (0.5 × 3.0)	9	38	−	−	−
11	0.1 (0.1 × 1.0)	2.0 (1.0 × 2.0)	8	72	+	+	−
12	2.0 (1.0 × 2.0)	2.0 (1.0 × 2.0)	8	45	−	+	−
13	0.1 (0.1 × 1.0)	2.0 (1.0 × 2.0)	4	57	−	+	−
14	—	2.0 (1.0 × 2.0)	7	32	−	+	+
15	0.1 (0.1 × 1.0)	2.0 (1.0 × 2.0)	8	48	−	−	+
16	0.1 (0.1 × 1.0)	2.0 (1.0 × 2.0)	7	36	+	+	+
17	—	2.0 (1.0 × 2.0)	8	34	−	−	+
18	0.1 (0.1 × 1.0)	3.0 (1.0 × 3.0)	6	72	+	+	−
19	0.1 (0.1 × 1.0)	3.0 (1.0 × 3.0)	4	44	−	−	−
20	0.1 (0.1 × 1.0)	3.0 (1.0 × 3.0)	7	63	−	−	+
21	0.1 (0.1 × 1.0)	3.0 (1.0 × 3.0)	5	51	−	+	+
22	0.5 (0.5 × 1.0)	3.0 (1.0 × 3.0)	8	37	−	+	+
23	0.1 (0.1 ×.1.0)	3.0 (1.0 × 3.0)	9	51	−	−	−
24	0.0 (0.0 × 0.0)	3.0 (1.0 × 3.0)	8	66	+	−	−
25	0.1 (0.1 × 1.0)	3.0 (1.0 × 3.0)	7	45	−	+	+
26	0.1 (0.1 × 1.0)	3.0 (1.0 × 3.0)	6	44	−	+	+

[a] For each case, lumican score (proportion of positive cells × estimated average intensity), determined as indicated in "Materials and Methods," is given for normal and tumor components. —, compartment not present within studied section; NS, axillary nodal status.

of axillary nodes, as specified in "Materials and Methods"). Increased lumican expression was associated with higher tumor grade ($P < 0.04$), younger patient age ($P < 0.04$), and ER status ($P < 0.05$). No correlation was observed between lumican expression and PR levels or nodal status. The conclusion that lumican expression is restricted to stromal cells was further supported by RT-PCR analysis of RNA extracted from several epithelial breast cancer cell lines (T47-D, T47-D5, MCF-7, MDA-MB-231, MDA-MB-468, and BT20) that did not show detectable levels of lumican mRNA (data not shown).

Analysis of Lumican mRNA Expression in Relation to Breast Tumorigenesis. To determine the relationship between the increased expression of lumican mRNA and breast lesions associated with increasing risk of invasive cancer, a range of tissues containing normal breast tissue, benign proliferative lesions, preinvasive ductal carcinoma *in situ*, and invasive carcinoma were studied by RT-PCR assay. This was performed on 1 mg of total RNA, extracted from histologically defined frozen sections. Three independent PCR experiments were performed that gave similar results as presented in Fig. 2. A lumican-corresponding band was observed in all of the invasive tumor samples but was undetectable or present only at low levels in all of the normal samples, consistent with the results from *in situ* hybridization, as detailed above. Between these two extremes, a very faint band or no band was detectable in proliferative disease without atypia lesions, but high levels comparable with those seen in invasive tumors were present in most pure ductal carcinoma *in situ* samples. The differences observed were not attributable to differences in input cDNAs, as shown by the intensity of *GAPDH* signals in each samples.

SDS-PAGE Detection of Lumican Protein in Breast Tumors. To characterize the lumican protein in breast tumor tissue, total proteins were extracted from three cases expressing lumican mRNA, as assessed by *in situ* hybridization. Western blot analysis of these proteins using an antilumican serum revealed that lumican protein is effectively expressed in breast tumor tissue (Fig. 3). Because several forms of lumican have been described (11), which differ by their glycosylated chains by sizes ranging from M_r 65 to 150,000, the effect of keratanase II and endo-β-galactosidase, which are capable of degradating sulfated and nonsulfated polylactosamine chains, respectively, was also investigated (Fig. 3). Keratanase II treatment had little, if any, effect on the size of tumor-derived lumican, whereas the endo-β-galactosidase reduced the size of the lumican to a relatively homogeneous component of M_r 55,000. This corresponds in size to the protein core of lumican observed in adult articular cartilage, which is devoid of keratan sulfate or polylactosamine chains (11). Thus, the tumor lumican appears to possess nonsulfated or poorly sulfated polylactosamine chains rather than the more highly sulfated keratan sulfate. Furthermore, there is no evidence of proteolytic degradation of lumican occurring in the tumor because the protein core size corresponds to that expected for the intact molecule following endo-β-galactosidase treatment.

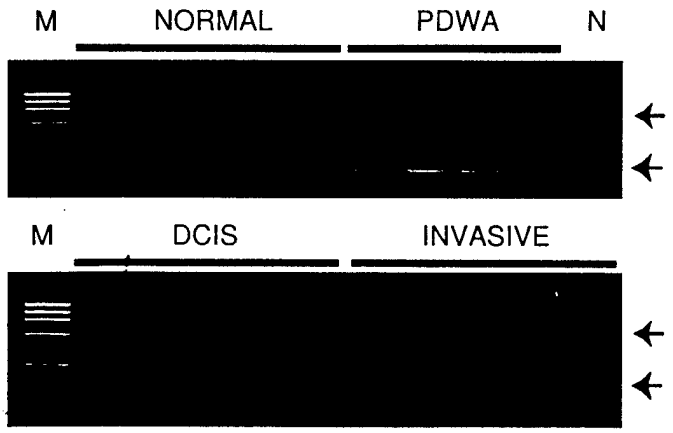

Fig. 2. RT-PCR analysis of lumican and GAPDH mRNA expression in normal samples from reduction mammoplasties (*NORMAL*) and samples from proliferative disease without atypia (*PDWA*; *top*), ductal carcinoma *in situ* (*DCIS*), and invasive ductal carcinoma (*INVASIVE; bottom*). PCR products were mixed before separation on 2% agarose gels and staining with ethidium bromide. *Black arrow*, product corresponding to lumican; *gray arrow*, product corresponding to *GAPDH*. *Lanes M*, molecular weight marker (ϕ×174 RF DNA/*Hae*III fragments; Life Technologies, Inc., Grand Island, NY). *Lane N*, negative control, no cDNA added during the PCR.

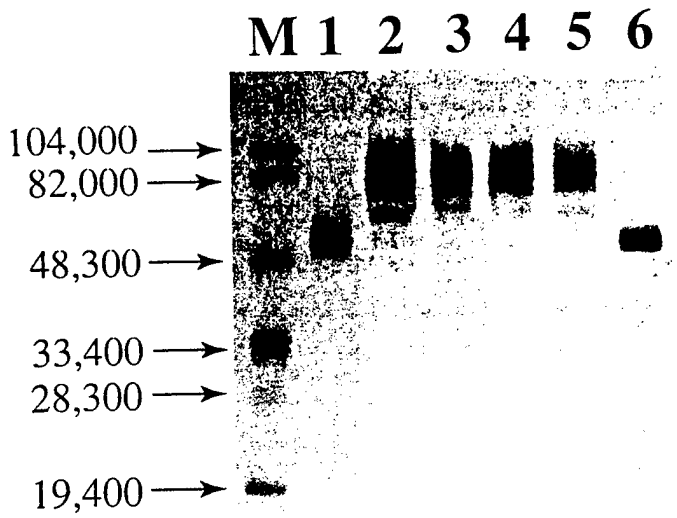

Fig. 3. Immunoblotting analysis of lumican. Proteins were extracted from three human breast tumors and lumican present in the extracts was analyzed by SDS-PAGE and immunoblotting as described in "Materials and Methods." An adult cartilage extract (*Lane 1*) and three breast tumor extracts (*Lanes 2–4*) were analyzed directly. One breast tumor extract was analyzed following treatment with keratanase II (*Lane 5*) and endo-β-galactosidase (*Lane 6*). *Left*, migration position of molecular weight markers.

Discussion

The data presented here demonstrate the presence and increased expression of lumican mRNA in human breast tumors relative to normal breast tissue. Lumican is a major constituent of the corneal stroma that is thought to participate in the acquisition of corneal transparency by regulating collagen fibril diameter and interfibrillar spacing (13, 14). Lumican expression has already been described in several other human tissues, including skin, articular cartilage, heart, placenta, skeletal muscle, kidney, and pancreas (11). This is, however, to our knowledge, the first observation of lumican expression in breast tissue, as well as its possible deregulation in tumorigenesis. Lumican belongs to the family of small interstitial leucine-rich proteoglycan proteins. Like other members of this family (decorin, biglycan, and fibromodulin), the lumican core protein contains a central region of leucine-rich repeats flanked at either side by a disulfide-bonded domain. The central region of the molecule possesses four asparagine residues capable of participating in *N*-linked glycosylation. Modification of *N*-linked glycosaccharides by sulfation of their polylactosamine units led to the classification of lumican as a keratan sulfate proteoglycan. Small keratan sulfate proteoglycans have been shown to organize collagen fibrils in extracellular matrix and may, therefore, be involved in the maintenance of tissue stromal structure (15, 16). However, other potential functions include the ability to influence cell adhesion or cell growth through interactions with growth factors (17, 18). Modifications of extracellular matrix components during tumor progression have been extensively reported, and the potential importance of proteoglycans in particular has also been underlined in both colon and breast cancer (19–24). In colon cancer, increased stromal expression of both large and small proteoglycans has been observed in tumor stroma, whereas ectopic expression of decorin in a colon tumor cell line reduced growth and tumorigenic potential. In breast cancer, different proteoglycans are distinct in their pattern of expression (21). The large proteoglycan versican has been shown to be prominent in the fibrous stroma within invasive tumor, as compared to surrounding normal tissue stroma (24). This contrasts with the pattern of expression of small leucine-rich chondroitin and dermatan sulfate proteoglycans, which were predominantly localized by immunocytochemistry to surrounding normal tissues and were absent from the invasive tumor stroma (24).

Our results suggest that increased expression of lumican can occur in tumor stroma in a similar pattern to that of versican. We have also found, albeit in a small cohort, an association between increased lumican mRNA expression and high tumor grade, younger patient age, and low ER levels, all of which are factors that are associated with increased tumor aggressiveness. Further study is needed to confirm this in a larger cohort and to distinguish whether this reflects a cause or an effect of increased tumor progression.

Here, we show that lumican protein is present in breast tumors mainly in its nonsulfated polylactosamine form. Such a nonsulfated form of lumican has already been described in noncorneal tissues, including articular cartilage and aorta (11, 25). The putative functions of lumican in noncorneal tissues, as well as the possible influences of its glycosylation state, remain to be established. In cornea, a conversion from nonsulfated polylactosamine chains to keratan sulfate chains, concurrent with eye opening, is suspected to contribute directly to corneal transparency (13, 26). Conversely, poorly sulfated chains replace highly sulfated ones during pathological conditions of the cornea including stromal inflammation and scarring (27, 28). Funderburgh *et al.* (28) recently demonstrated that, in contrast to the highly sulfated form of lumican, the nonsulfated form of the protein promotes macrophage attachment and spreading. This action is thought to be mediated through a high-affinity receptor for lumican, expressed in a constitutive manner by macrophages and different from the already described scavenger receptor or from receptors for other extracellular matrix molecules. This receptor recognizes the lumican protein both in glycosylated and deglycosylated forms through structures that can be masked by keratan sulfate chains. Poorly sulfated lumican could, therefore, act to localize macrophages in regions of inflammation. One could speculate that increased expression of lumican in its nonsulfated state in breast cancer may be a mechanism that encourages macrophage adhesion and localization within the tumor. This increased macrophage concentration could in turn influence angiogenesis and prognosis through the production of tumor-associated macrophage cytokine production (29, 30).

In conclusion, the detection of lumican in the stromal reaction within breast carcinoma suggests than this proteoglycan may have a role in breast tumorigenesis and progression. Further studies are needed to determine the exact function of lumican in breast tissues and how changes in its expression and in its glycosylation pattern modify properties of the extracellular matrix or the adjacent tumor cells.

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APPENDIX F

LUMICAN AND DECORIN ARE DIFFERENTIALLY EXPRESSED IN HUMAN BREAST CARCINOMA

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Running title: Lumican expression in breast cancer.

Key words: Lumican, decorin, small leucine-rich proteoglycan, breast cancer, tumor progression.

Footnotes:

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3 The abbreviations used are: SLRP, small leucine rich proteoglycan; RT-PCR, reverse transcription-polymerase chain reaction; H&E, Hematoxylin/Eosin; ER, estrogen receptor; PR, progesterone receptor; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. NS = not significant.

Abstract

We have previously shown that lumican is expressed and increased in the stroma of breast tumors. We have now examined lumican expression relative to three other members of the small leucine-rich proteoglycan gene family in 45 normal and neoplastic breast tissues. Western blot study showed that lumican protein is highly abundant relative to decorin, while biglycan and fibromodulin are only detected occasionally in both normal and tumor tissues (n=6+9). Analysis of matched tissues by RT-PCR (n=15) and Western Blot (n=12) showed that lumican mRNA and protein is significantly increased in tumors cases ($p<0.05$) while decorin shows the opposite trend with a decrease in tumor relative to normal tissue. This observation was confirmed by in-situ hybridization (n=15) where both genes are found to be expressed by similar fibroblast-like cells adjacent to epithelium, but lumican mRNA is increased ($p=0.021$) while decorin mRNA is reduced ($p=0.028$) in neoplastic relative to normal stroma. Further evidence of altered lumican expression is manifested by discordance in some regions of tumors but not normal tissues, between lumican mRNA and protein localization. We conclude that lumican is the most abundant of these proteoglycans in breast tumors and that lumican and decorin are inversely regulated in association with breast tumorigenesis.

Introduction

The development and progression of breast carcinoma is caused by alterations in the expression of multiple genes, most of which are responsible for normal physiological pathways and the necessary cellular interactions to support these functions within the mammary gland. These include alterations in the interactions between the epithelial and stromal cells, which are manifested in tumors by well recognized morphological changes known as the stromal reaction [1]. The influence of such alterations in stromal-epithelial interactions may influence the risk of transformation of the breast epithelial cell and very early steps in tumorigenesis, as has been recently proposed in other systems [2]. However, the net effects of these alterations in the stroma on later stages of tumor progression is unresolved [3]. For example a net positive influence on tumor progression might occur through increased availability of stimulatory growth factors or vascular supply through angiogenesis. Alternatively a net negative influence could occur through the release of inhibitory growth factors or the establishment of a physical barrier to invasion.

Resolution of this issue is complicated by the recognition that behind the plain morphology of the fibroblast and the bland collagen fibres observed in the stroma of a histological breast tissue section, lies a highly complex tissue. This includes a variety of different types of fibroblasts [4, 5] and a range of proteins, glycoproteins and proteoglycans which may play a role in this aspect of tumor biology. We have recently extended this list by identifying lumican, a member of the small leucine-rich proteoglycans (SLRP's) as an mRNA that is expressed in the stroma of normal breast tissues and overexpressed in invasive carcinomas [6]. To explore further the potential role of lumican and related genes in breast tumor progression, we have now examined the expression of lumican relative to that of other members of the SLRP family, decorin, biglycan and fibromodulin, at both mRNA and protein level in 45 normal and neoplastic breast tissues.

Materials and methods

Human breast tissues

All breast tumor cases used for this study were selected from the NCIC-Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). As has been previously described [7], tissues are accrued to the Bank from cases at multiple centers within Manitoba, rapidly collected and processed to create matched formalin-fixed-paraffin-embedded and frozen tissue blocks with the mirror image surfaces oriented by colored inks. The histology and cellular composition of every sample in the Bank is interpreted in Hematoxylin/Eosin (H&E) stained sections from the face of the former tissue block. This information is available in a computerized database along with relevant pathological and clinical information and is used as a guide for selection of cases for study.

For the initial study to broadly compare expression of different members of the SLRP gene family a mixed cohort was selected from the Tumor Bank to include 9 different invasive carcinomas, 3 normal tissue samples from patients with cancer and 3 normal tissues from normal patients without cancer. The invasive tumors included a range of tumor types (5 ductal, 3 lobular and 1 tubular carcinoma), grades (4 high, 1 moderate, 4 low grade) and estrogen receptor levels (3 ER <10 fmol/mg, 3 ER 10-20, 3 ER 39-169) and total stromal fractions ranging from 50-95% of the cross sectional area. The mean patient ages were 62, 70 and 28 years for each subgroup respectively.

For the subsequent studies to compare lumican and decorin expression a second more defined and homogeneous cohort of 15 cases was selected to provide matching primary tumor tissues and adjacent normal tissue blocks. This cohort included only invasive ductal carcinomas and was primarily selected to ensure availability of histologically confirmed normal and tumor frozen tissue blocks, equivalent stromal content in each site, as far as was possible between cases (mean stromal area $^{(sd)}$ in tumor tissues = $68^{(10)}$ %, adjacent normal tissues = $89^{(6)}$ %) and to incorporate cancer cases from both post-menopausal patients (6 cases mean$^{(sd)}$ = $76^{(7)}$ years), peri-menopausal patients (6 cases mean$^{(sd)}$ = $55^{(5)}$ years) and pre-menopausal patients (6 cases mean$^{(sd)}$ = $44^{(3)}$ years).

Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) and immunoblotting

Total proteins were extracted from frozen tissue sections cut from frozen tissue blocks and from the face immediately adjacent to the surface of the matching paraffin block from which sections were cut for

pathological assessment and in-situ hybridization. For the first cohort of cases, the numbers of tissue sections used for each extraction was varied empirically according to the area of the tissue block to achieve equivalent volumes of tissue for the extraction, which was done using 4M guanidinium chloride in the presence of proteinase inhibitors as described previously [8]. For the second cohort of matching tissue samples, the same number of frozen sections (20 x 20 micron) was cut from each tissue block and protein extraction performed with equivalent volumes of extraction buffer. Proteins present in equivalent volumes of extracts were analyzed by SDS/PAGE and immunoblotting, using anti-peptide antibodies specific for the carboxyl-terminal regions of lumican, decorin, fibromodulin and bigyclan [8, 9]. Protein signals were detected by chemiluminescence and photographed prior to quantitation by video-image analysis and densitometry using an MCID M4 software (Imaging Research, St Catherines Ont.). All signals were adjusted with reference to control cartilage samples run with each blot and in the second series of matched tissue samples with reference to the measured cross sectional area of the tissue block to control for equivalent loading. Additional analysis was performed on all signals after further adjustment for relative stromal content of the tissue sections assessed in adjacent H&E sections.

Immunohistochemistry

Immunohistochemistry was performed on paraffin sections using the same antibody to lumican as used for immunoblotting. Sections (5 micron thick) were obtained from paraffin embedded tissue blocks matching the frozen tissue blocks of those cases used for RT/PCR and protein analysis. After deparaffinizing, clearing and hydrating in TBS buffer (Tris Buffered Saline, pH 7.6) the sections were pre-treated with hydrogen peroxide, 3% for 10 minutes to remove endogenous peroxidases and non-specific binding was blocked with normal swine serum 1:10 (Vector Laboratories S-4000). Tris buffered saline (TBS) was used between steps to rinse and as a diluent. Primary antibody to lumican was applied at a 1:400 dilution overnight at 4 °C followed by biotinylated secondary swine anti-rabbit IgG, 1:200 (DAKO) for 1 hour at room temperature (R.T.). Tissue sections were incubated 45 minutes at R.T. with an avidin/biotin horseradish peroxidase system (Vectastain ABC Elite, Vector Lab.) followed by detection with DAB (diaminobenzidine), counterstaining with Methyl Green, 2% and mounting. A positive tissue control (colon mucosa) and a negative reagent control (no primary antibody) were run in parallel. Immunostaining pattern and intensity was assessed by light microscopic visualization.

RT-PCR analysis

Total RNA was extracted from frozen tissue sections of 6 normal and 9 tumor tissues using a small scale RNA extraction protocol (Tri Reagent, Sigma) [12]. Total RNA, 0.1 ug was reversed transcribed with random hexamer primers in a final volume of 40 ul and 1.5 ul of the reaction mixture subsequently amplified by PCR as previously described [6]. PCR primers used corresponded to the lumican gene [8], sense (5'-GGCTGAAAGAGGATGCTGT-3') and antisense (5'-TGGTTTCTGAGATGCGATTG-3'), the decorin gene [10] sense (5'-AAATGCCCAAAACTCTTCAG-3') and antisense (5'- AAACTCAATCCCAACTTAGCC-3'), and to the ubiquitously expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene [11], sense (5'-ACCCACTCCTCCACCTTTG-3') and antisense (5'- CTCTTGTGCTCTTGCTGGG-3'). To amplify cDNA corresponding to lumican and decorin PCR was run for 30 cycles (1 minute at 95C, 1 minute at 52°C, and 2 minutes at 72°C). For amplification of GAPDH cDNA PCR consisted of 28 cycles (45 seconds at 93°C, 45 seconds at 54°C, and 1.5 minutes at 75°C). Fifteen microlitres of PCR product was analyzed by electrophoresis using 2% agarose gels and post-stained with ethidium bromide (1.0 ug/ml). Preliminary experiments were conducted with representative samples to establish the linear range for amplification of lumican, decorin, and GAPDH PCR products and to allow videodensitometry and semi-quantitative analysis of lumican and decorin signals relative to GAPDH. At least two independent PCR reactions for each gene were performed and analysed for each sample.

In-situ hybridization

Paraffin embedded 5 micron sections of breast tumor tissue were analysed by 'in situ' hybridization according to a previously described protocol. For lumican, the plasmid Lumi-398, that consisted of pCR 2.1 plasmid (Invitrogen, San Diego, Ca.), containing a 398bp portion of lumican cDNA, between bases 1332 and 1729 [6],was used as a template to generate UTP^{S35} labelled sense and antisense riboprobes using Riboprobe Systems (Promega, Madison,WI.) according to the manufacturer's instructions. For decorin, a 191 bp cDNA was first generated by RT-PCR from a breast tissue sample for probe synthesis using primers that corresponded to decorin (sense 5'-AAGGTTCCCTGGTTGTGA -3' and antisense 5'- GCGGTCATCAGGAACTTC-3') [10] and included an SP6 promoter sequence. The decorin PCR product was run on an agarose gel and purified with a GFX PCR gel band purification system (Pharmacia Biotech) and the DNA was dried down and redissolved in TE buffer, pH 8.0. As previously described 1.0 ug/ul of each cDNA was *in vitro* transcribed with UTP (S^{35})

and working sense and antisense probes were equalized by diluting in hybridization solution. Sections underwent pretreatment with triethanolamine/acetic anhydride and proteinase K. Approximately 30 ul of probe ($1x10^6$ cpm/ul) was applied to each section, covered, sealed and incubated overnight in a humid chamber at 42 - 44 C. After coverslip removal, sections underwent an RNase buffer treatment followed by several SSC buffer washes to remove any weakly bound non-specific label. After dehydration in ethanols, the dried sections were coated in NTB-2 Kodak emulsion, developed at various time intervals from 7 to 28 days and counterstained with Methylene Blue and Basic Fuchsin. Levels of lumican and decorin expression were assessed in normal and tumor regions by brightfield microscopic examination at low power magnification and with reference to the negative sense and positive control tumor sections. This was done as previously described [6] by scoring the estimated average signal intensity (on a scale of 0 to 3) and the proportion of stromal cells showing a positive signal (0, none; 0.1, less than one tenth; 0.5, less than one half; 1.0 greater than one half). The intensity and proportion scores were then multiplied to give an overall score. Regions with a score lower than 1.0 were deemed negative or weakly positive.

Microdissection and protein extraction analysis

To assess protein localization within regions of tumors, 2 cases were selected that showed marked and well defined regions within the same tissue section with discrepancies between mRNA and protein expression. This was determined by in-situ hybridization and immunohistochemistry in adjacent serial sections from paraffin tissue blocks. The mirror image frozen tissue blocks to these paraffin blocks were used for microdissection as previously described [12] and protein was extracted from these histologically defined regions as described above. Briefly, thin 5 micron frozen sections were cut from the face of the frozen tissue blocks and stained by H&E, and the relevant histological regions of approximately 1-2 mm^2 distinguished and confirmed by reference to the paraffin sections already studied. Multiple thick frozen sections (20 x 20 micron) were then cut, rapidly stained and microdissected at room temperature from each section in turn, and the microdissected tissue fragments frozen again prior to protein extraction.

Results

Identification of lumican as the most abundant SLRP in normal and neoplastic breast tissues

To determine the relative importance of altered lumican expression in breast tumorigenesis the expression of lumican protein was compared to that of 3 other members of the SLRP family, decorin, fibromodulin and biglycan by Western blot in a heterogeneous panel of 9 breast tumors and 6 normal tissues (Table 1, Figure 1). Lumican was highly abundant in all samples and in both neoplastic and normal tissues. A small but not significant increase was seen in mean level of lumican protein between these normal and tumor samples (mean$^{(sd)}$ optical density units; normal = 0.38$^{(0.07)}$, tumor = 0.41$^{(0.11)}$, p= ns, Mann Whitney test). However, this difference became significant after adjustment of the signals for the relative percentage area within the tissue section occupied by collagen and fat, where lumican is expressed and localized (mean$^{(sd)}$ tissue adjusted optical density units, normal = 0.43$^{(0.08)}$, tumor = 0.56$^{(0.15)}$, p= 0.026, Mann Whitney test). An apparent difference in level of lumican between normal samples from normal patients and normal samples adjacent to tumors was seen but in this case the difference did not persist when different stromal content of these samples was taken into account. Nevertheless, an increase in overall molecular weight and polydiversity was noted in normal tissues from older patients relative to younger patients.

Immunohistochemical study of lumican distribution within these same tissues performed using the same antibody showed that lumican was abundant throughout the collagenous stroma of both normal and tumor sections with prominent deposition around small vessels, breast duct and lobular structures. There was increased deposition within the collagenous stroma of tumors, in particular at the invasive margins and in areas of dense collagen within central regions of some tumors, compared to adjacent normal tissues (Figure 2C&D). However, in some cases there were distinct regions within the tumor sections containing loose stroma in which there was a notable absence of lumican.

In comparison decorin, although also present in most samples examined by Western blot, was much less abundant relative to the cartilage control. It should be noted that the decorin (in common with biglycan and fibromodulin) signals shown in figure 1 also required a 3 fold longer chemiluminescent exposure time (9 seconds) than that for lumican (3 seconds). However, there was a marked and significant decrease in decorin between normal and tumor samples (mean$^{(sd)}$ optical density units; normal = 0.19$^{(0.05)}$, tumor = 0.1$^{(0.1)}$, p= 0.026, Mann Whitney test). A similar trend was also found after adjustment of signals for stromal content

(mean$^{(sd)}$ tissue adjusted optical density units; normal = 0.21$^{(0.06)}$, tumor = 0.13$^{(0.14)}$, p= 0.07, Mann Whitney test). No difference was seen in signals between normal samples from normal and cancer patients. Fibromodulin expression was not detected in normal tissues and in only 3/9 tumors, where the presence of fibromodulin correlated with those tumors with the highest content of epithelial tumor cells. Biglycan was detected in 2/6 normal tissues and 3/9 tumors, where in contrast to fibromodulin its presence correlated directly with those tumors with the highest content of collagenous stroma.

Lumican and decorin are differentially expressed between normal and neoplastic tissues.

In order to examine further the distinct alterations in the expression of lumican and decorin, the mRNA and protein expression of both genes was examined by RT-PCR and Western blot in 15 and 12 cases respectively from the second case series, comprising matched normal and tumor samples (Table 2). In 9 cases sufficient tissue was available to allow quantitation on both mRNA and protein extracted from the same cases and adjacent sets of frozen sections.

Assessment of mRNA levels by semi-quantitative RT-PCR in 15 cases confirmed our previous observations, and lumican mRNA was found to be significantly elevated in tumors (p=0.048, Wilcoxon test). Lumican mRNA was also significantly elevated in estrogen receptor negative tumors (mean$^{(sd)}$ lumican optical density units; 0.30$^{(0.12)}$ vs 0.14$^{(0.09)}$, p=0.04, Mann Whitney test) and showed a trend towards elevation with high grade tumors (mean$^{(sd)}$, 0.27$^{(0.13)}$ vs 0.15$^{(0.07)}$, p=0.17 ns) on analysis of small subgroups within this series of tumors as previously defined [6]. At the same time, decorin levels showed a consistent but not significant trend (p=0.094, Wilcoxon test) towards lower levels in tumor relative to normal tissues (Table 2).

In keeping with the pattern of mRNA expression, the mean lumican protein signal assessed by Western Blot was also higher in 9/12 tumors relative to normal tissues (mean$^{(sd)}$ optical density units, normal = 0.66$^{(0.46)}$, tumor = 1.033$^{(0.53)}$, p=0.043 Wilcoxon test). Once again, in contrast to this, decorin protein was lower in 7/12 tumors relative to normal tissues but the differences were not significant (mean$^{(sd)}$ optical density units, normal = 0.168$^{(0.15)}$, tumor = 0.123$^{(0.132)}$, p= ns, Wilcoxon test). The trend in both protein and mRNA expression between normal and neoplastic tissues correlated in 7/9 cases for lumican but in only 4/7 cases for decorin. These contrasting patterns of lumican and decorin expression also persisted after standardisation of both RT-PCR and Western blot signals for relative stromal content.

To examine this issue directly within tissues, in situ hybridization was conducted on the initial series of

15 cases (Table 1, Figure 3). As previously shown, prominent lumican mRNA expression was detected using an antisense probe, in stromal fibroblast like cells within the tumor and immediately adjacent to invasive tumor cells. By comparison, lumican expression was lower in normal tissues, where these were present in the same section adjacent to the tumor (5 cases) and in the 6 unmatched normal cases (Figure 3B). The level of expression of lumican was evaluated within normal and tumor compartments using a semi-quantitative approach as detailed in the Materials and Methods section. Lumican was found to be expressed at low levels (=< 1) within the collagenous stromal tissues associated with normal ducts and lobules in all but 2/11 normal tissues, whereas significantly higher expression was evident (>1) in stromal fibroblast-like cells within the collagenous stroma of 7/9 invasive tumors ($p=0.022$, Fisher's Exact Test). Decorin mRNA was seen in the same regions of both tumor and normal tissues, and appeared to be expressed by the same stromal fibroblast-like cells as far as could be determined by comparison of the signals of lumican and decorin in serial sections (Figure 3C). However, in direct contrast to lumican, decorin was again found to be expressed at high levels (> 1) in 7/11 normal tissues but in only 1/9 invasive tumors ($p=0.028$, Fisher's Exact Test).

Lumican mRNA and protein expression can occur in different regions within breast tumors

As noted above, in some tumors there were distinct regions, up to 2 mm in area, in which there was a complete absence of lumican detectable by immunohistochemistry (Figure 2 C&D). However, the same regions showed high expression when examined for lumican mRNA by in-situ hybridization in adjacent sections (Figure 2A&B). Similarly, other areas showed strong staining for lumican protein but low levels of mRNA. To explore the possibility that the absence of lumican expression detected by immunohistochemistry might be due to the conformation of the native protein or the binding of lumican to other proteins, resulting in the masking of the carboxy-terminal epitope, specific areas measuring approximately 1 mm^2 each were microdissected from frozen sections of 2 tumors and lumican protein assessed under denaturing conditions by SDS/PAGE and Western blot. In both cases, those regions with high mRNA expression and negative by immunohistochemistry were also negative by Western blot, while areas showing very low mRNA expression but strong staining by immunohistochemistry were positive by Western blot (Figure 4).

Discussion

We have shown that lumican is the most abundant proteoglycan in comparison with several other members of the family of small leucine-rich proteoglycans (SLRP's) in breast cancer. We have also extended our previous observations [6], based on the detection of lumican mRNA, in showing that the total lumican protein is also increased in breast tumors relative to adjacent normal tissues. Our results also demonstrate that this pattern of upregulation of lumican in relation to breast tumorigenesis is distinct from that of the closely related decorin gene, which is inversely regulated and reduced at both mRNA and protein levels, in tumor relative to adjacent normal tissue. Finally, we have shown that lumican expression in tumors may also be associated with abnormal distribution within the stroma manifested by discordance between mRNA and protein deposition within subregions of breast tumors.

The family of small interstitial leucine-rich proteoglycans share common features, including a central region of leucine rich repeats bounded by flanking cysteine residues and localization in the extracellular matrix. However, it is now recognized that they can be separated into three subgroups of genes, that include decorin and biglycan, lumican and fibromodulin, epiphycan and osteoglycin, that are distinguishable both by amino acid homologies and also gene structure [13]. Decorin, probably the best studied of these genes, is known to interact with a variety of extracellular matrix molecules and growth factors and has been shown to be capable of influencing collagen fibril growth and assembly both in-vitro and in-vivo [14-16]. Decorin may also influence tumor cell growth through indirect effects on the availability of growth factors from the extracellular matrix or directly through activation of the EGF receptor and induction of the p21 cell cycle inhibitor [17, 18]. In contrast less is known about lumican and other SLRP's. However, in-vitro and in-vivo data indicate that lumican is also important in the regulation of collagen fibril assembly [16, 19]. This view is supported by recent observations based on mice with homozygous deletion of the lumican gene where loss of corneal transparency and increased skin fragility is associated with disorganized and loosely packed collagen fibers related to increased and irregular fibril size, and interfibrillar spacing, as viewed by light and electron microscopy [20].

The observation that lumican is highly abundant compared to other SLRP's in breast tumors cannot be interpreted to mean that it is necessarily the most important. This is underscored by the recent demonstration that although decorin is apparently more abundant than versican in prostate cancer tissue, only increase in the larger chondroitin sulphate proteoglycan versican correlates with grade and inversely with progression-free

survival in prostate cancer [21]. Similarly, the increase in lumican seen here in association with breast tumorigenesis may be less important than the parallel decrease in decorin. The implications of these alterations in expression await experiments to determine their functional effects. However, increased lumican expression is associated with several parameters indicative of more biologically aggressive tumors [6] indicating that the relative changes in different SLRP expression may have a functional effect on tumor progression. It is possible to speculate that both induction of lumican and decrease in decorin in stromal fibroblasts within the invasive tumor may represent a positive host response to abrogate the disorganisation of collagen within the tumor stroma, encourage macrophage localization [22], and inhibit the growth of epithelial cancer cells through the increased availability of growth factors inhibitory to breast epithelial cell growth [18, 23]. Alternatively, these alterations may represent a negative host response contributing to tumor progression. Increased lumican mRNA expression may reflect a response to locally increased proteolysis or altered deposition of the lumican protein that is the cause of the disorganization of the collagenous stroma, that in turn facilitates tumor cell invasion. Similarly, a decrease in decorin may remove an inhibitory effect on epithelial tumor cell growth though repression of p21 [18, 24]. A role for and the distinction between these opposing potential effects will clearly require further study.

Although we have shown that lumican protein is more abundant in tumors, the differences observed by both immunohistochemistry and Western blot are not as marked as those seen at the level of mRNA expression. While differences in the assays may account for some of this discrepancy, it is clear that it may also be attributable to the discordance that can also exist between lumican mRNA and protein expression detected by in-situ and immunohistochemical techniques respectively, within the same regions of breast tumor stroma. A similar discordance between mRNA and protein expression has been previously observed in the course of studies on lumican and other large and small proteoglycans in different tissues. For example, in corneal development in the chicken, the mRNA levels for lumican and decorin do not always reflect the rate of synthesis of the corresponding proteins and the efficiency of translation of lumican varies over time [25]. Similar discordance between aggrecan and versican mRNA and protein has been seen in normal tendon [26], between decorin and biglycan mRNA and protein localisation in normal and reactive gastric mucosa [27], and in regions of cartilage matrix around vascular channels and the growth plates of long bones in normal cartilage [28]. In this latter instance, the discordance was attributed to high rate of breakdown and removal at these sites. This

conclusion is supported by studies on endothelial cells which show that growth factors such as bFGF can not only increase both biglycan transcription and protein synthesis but also the corresponding rate of proteolysis [29]. The absence of protein could also reflect masking of the epitope by conformational changes in the native protein, by changes in post-translational modification or by binding to another protein. Alternatively this could reflect reduced translation, increased breakdown, or failure to bind within the immediate stroma and rapid translocation of the protein to adjacent areas of the tissue. Our microdissection experiments, applied to small regions where lumican mRNA is highly expressed suggest that the corresponding protein is truly absent in these areas and that epitope masking due to conformation or binding proteins is an unlikely explanation for the observation. However it could also be the case that the necessary binding sites are not available in the immature stroma associated with rapid growth of tumors and that this allows translocation of newly synthesized lumican to binding sites in adjacent tissue.

The direction and the reciprocal nature of the changes in the expression of lumican and decorin occurring apparently in the same fibroblast-like cells in breast tissue stroma is intriguing. While lumican has not been previously studied in human tumors other than breast cancer, expression of decorin mRNA and proteoglycans incorporating chondroitin sulphate epitopes have been shown to be increased in colon, prostate and basal cell carcinomas [30-32]. However, similar immunohistochemical studies of breast tumors using monoclonal antibodies raised against chondroitin sulphate and dermatan sulphate small proteoglycan have shown reduced decorin expression within invasive as compared to surrounding normal stroma [33], consistent with our findings. Decorin and other SLRP's are known to be independently regulated and mutually exclusive [28] and compensatory changes in expression between different SLRP's have been observed [34]. However this appears to be usually manifested by genes within subgroups of the SLRP family. At the same time, reciprocal changes in expression between lumican and decorin have not been described in lumican or decorin 'knockout' mice [14, 20]. The factors that influence altered expression of these genes in breast tumor stroma remain to be elucidated.

In summary, we have shown that lumican is highly abundant relative to decorin, biglycan and fibromodulin in normal and neoplastic breast tissues. We have also shown that increased lumican protein expression and altered regional localization occur in breast tumors and that different and reciprocal alterations in expression occur between lumican and decorin. The functional significance and role of alterations in these stromal proteoglycans in breast tumorigenesis and progression remains to be determined.

Figure Legends.

Figure 1. Immunoblotting study of lumican, decorin, biglycan and fibromodulin protein expression in human breast tumors (lanes 1-9), normal tissues from normal patients (lanes 10-12) and normal tissues adjacent to carcinomas (lanes 13-15). All protein samples were extracted from sets of frozen tissue sections bracketed by sections assessed by H&E stain and light microscopy to confirm content. Chemiluminescent signals for decorin, biglycan and fibromodulin required 3 fold longer exposure times than that for lumican. Molecular markers (left) and cartilage control sample (right) are present in all panels.

Figure 2. In-situ hybridization and immunohistochemical study showing regional discordance in lumican mRNA (panels A & B) and protein expression (panels C & D) displayed in adjacent sections in breast tumors. Panels A and C show overall pattern of mRNA (A, black signal) and protein (C, brown staining) within a tissue section (0.4 x 0.8 cms in size) that includes regions of in-situ and invasive tumor (upper left and upper middle) and adjacent normal tissue (lower left and lower right). Panels B & D show a detailed microscopic view (original magnification x400) of the cellular localization of mRNA and protein within a small region at the invasive edge within the same section (tumor component at left, normal component at right)

Figure 3. Lumican and Decorin mRNA expression detected by in-situ hybridization within a breast tumor section. Panel A (H&E section) shows the histology including the invasive tumor (upper area), the tumor margin (middle) and adjacent normal tissue including lobular-ductal units (lower area). Lumican expression (panel B) is high within the tumor and tumor margin and lower in the normal fat and collagenous stroma adjacent to the normal lobules. Decorin (panel C) shows high expression in the normal stroma adjacent to normal lobules and reduced expression in the tumor. Original magnification x400.

Figure 4. Lumican protein expression detected by Western blot (lower panel) in microdissected subregions within two breast tumor sections (upper panels, scale bar = 5mm). The mRNA and protein signals were detected by in-situ hybridization (ISH) and immunohistochemistry (IHC) in each region in adjacent sections. They were assessed as follows; tumor A region ISH/IHC, 1 = ++/-, 2 = -/++, 3 = +/++ (remainder of section); tumor B region ish/ihc, 4 = +/-, 5 = -/++, 6 = ++/++. C = cartilage control.

Table Legends.

Table 1. Clinical-pathological-histological features and expression of SLRP's in the first cohort of breast tissues.

Age= patient age in years; Type = tumor type (DN – invasive ductal NOS, IL – invasive lobular, TU – tubular), GR = Nottingham grade, ER = estrogen receptor level, fmols/mg, area = area of tissue section in cm2, epi = normal or neoplastic lobular/ductal epithelial component, coll = collagenous stroma component, fat = fat stroma component, Lum = lumican, Dec = decorin, Big = biglycan, Fib = fibromodulin, N-Lum,T-Lum, N-Dec, T-Dec = lumican or decorin signals in areas of normal or tumor stroma. For each case, lumican score (proportion of positive cells x estimated average intensity), determined as indicated in the Materials and Methods section, is given for normal and/or tumor components; (-) compartment not present within the studied section.

Table 2. Clinical-pathological-histological features and expression of lumican and decorin in second cohort of matched normal and tumor samples.

Columns as described in table 1 except for Western blot and RT-PCR lumican and decorin levels expressed as H, signal higher in tumor than normal section; L,signal lower in tumor than in normal section, or =, signal is equivalent levels in both.

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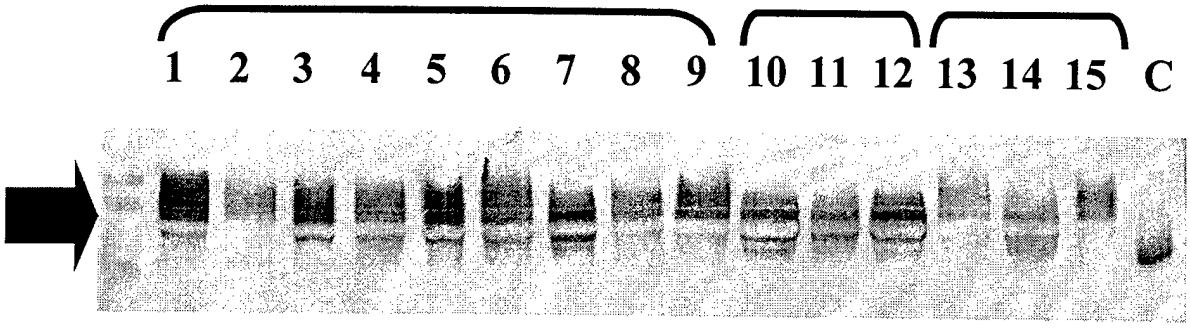
Table 1.

					Section Composition				Western Blot				In-situ Hybridization			
Study #	Age	Type	GR	ER	Area	epi	coll	fat	Lum	Dec	Big	Fib	N-Lum	T-Lum	N-Dec	T-Dec
1	71	IL	5	39	0.5	5%	55%	40%	0.62	0.10	0.05	0.00	1.5	1	2	0.5
2	45	DN	8	6.4	0.63	25%	15%	60%	0.23	0.01	0.00	0.00	-	0.75	-	0.25
3	57	TU	4	11.5	0.525	10%	20%	70%	0.43	0.05	0.00	0.00	0	2	2	1
4	80	DN	8	1	0.81	30%	65%	5%	0.37	0.05	0.11	0.00	-	1.5	-	0.75
5	66	IL	5	19.5	0.9	10%	40%	50%	0.49	0.03	0.00	0.00	0.05	1.5	0.25	0.25
6	72	DN	6	169	0.9	50%	40%	10%	0.42	0.07	0.00	0.01	0.5	1	1	0.5
7	51	DN	9	0.3	0.54	35%	15%	50%	0.46	0.15	0.00	0.02	0.5	1.5	2.5	0.5
8	66	DN	8	136	0.35	50%	50%	0%	0.28	0.03	0.00	0.16	-	3	-	0.5
9	48	IL	5	10.3	0.6	10%	65%	25%	0.43	0.39	0.04	0.00	-	0.05	-	0.25
10	68				0.2	10%	70%	20%	0.39	0.22	0.01	0.00	0.05	-	0.5	-
11	75				0.52	20%	50%	30%	0.37	0.22	0.00	0.00	0.75	-	2.5	-
12	66				0.15	10%	70%	20%	0.48	0.13	0.02	0.00	0	-	0.25	-
13	23				0.72	5%	60%	35%	0.26	0.19	0.00	0.00	0.75	-	0.75	-
14	37				0.55	10%	50%	40%	0.39	0.12	0.00	0.00	0.5	-	1.5	-
15	23				0.6	5%	80%	15%	0.40	0.25	0.00	0.00	1	-	2	-

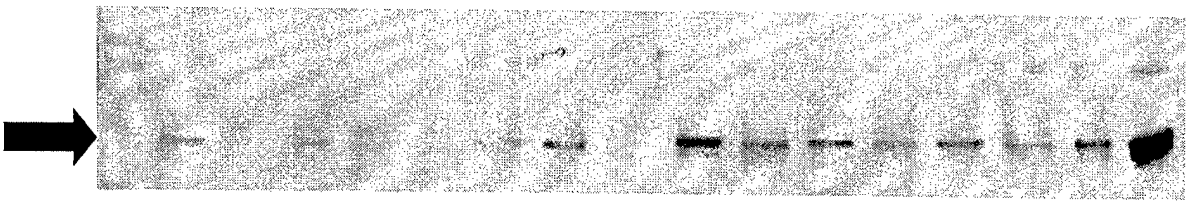
Table 2

				Normal section composition				Tumor section composition				Western Blot		RT-PCR	
Study#	Age	Grade	ER	area	epi	coll	fat	area	epi	coll	fat	Lum	Dec	Lum	Dec
16	69	9	1.7	1.08	15%	80%	5%	1.12	30%	50%	20%	=	L	H	H
17	45	9	0.7	0.50	10%	40%	50%	0.91	20%	30%	40%	H	L	H	L
18	44	5	2.7	1.20	20%	60%	20%	0.75	25%	65%	10%	H	H	H	H
19	44	7	6.8	0.24	5%	20%	75%	0.84	40%	30%	20%	H	H	H	-
20	73	6	0	0.75	25%	25%	50%	0.66	30%	70%	0%	H	H	-	-
21	86	8	39	0.96	10%	10%	80%	0.72	30%	40%	40%	H	L	H	-
22	39	7	3.5	0.77	10%	40%	50%	0.60	35%	50%	15%	H	L	H	L
23	49	7	8.6	0.75	5%	15%	80%	0.70	35%	60%	5%	H	H	H	L
24	83	7	2.2	1.08	5%	25%	70%	0.40	30%	60%	10%	H	H	-	-
25	75	7	159	1.33	10%	20%	70%	0.81	20%	55%	25%	H	=	-	-
26	71	9	47	0.40	5%	10%	85%	0.70	50%	40%	10%	H	L	H	L
27	41	8	6.4	1.20	10%	60%	30%	2.16	25%	45%	30%	L	L	H	H
28	52	8	11.1	0.55	10%	50%	40%	0.60	35%	50%	15%	-	-	L	H
29	54	8	2.8	0.15	10%	80%	10%	0.60	70%	25%	5%	-	-	-	H
30	56	9	0	0.32	5%	70%	25%	0.70	20%	20%	60%	-	-	H	L
31	64	5	55	0.40	5%	10%	85%	0.65	35%	35%	30%	-	-	L	L
32	55	6	60	0.40	15%	60%	25%	0.32	25%	60%	15%	-	-	H	L
33	51	9	31	0.24	5%	45%	50%	0.54	55%	35%	10%	-	-	H	L

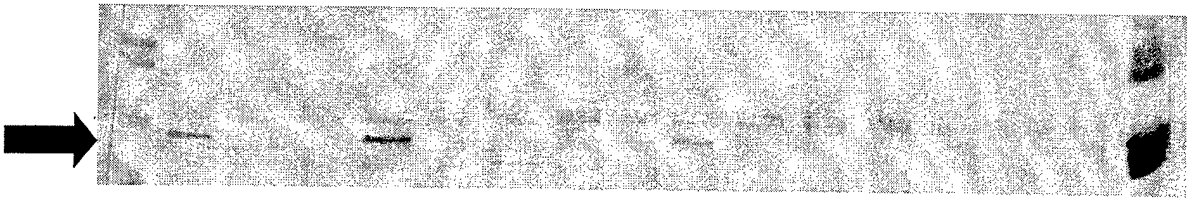
Figure 1



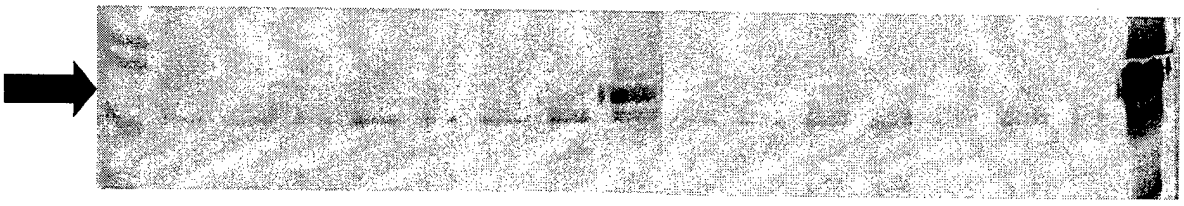
LUMICAN



DECORIN



BIGLYCAN



FIBROMODULIN

Figure 2

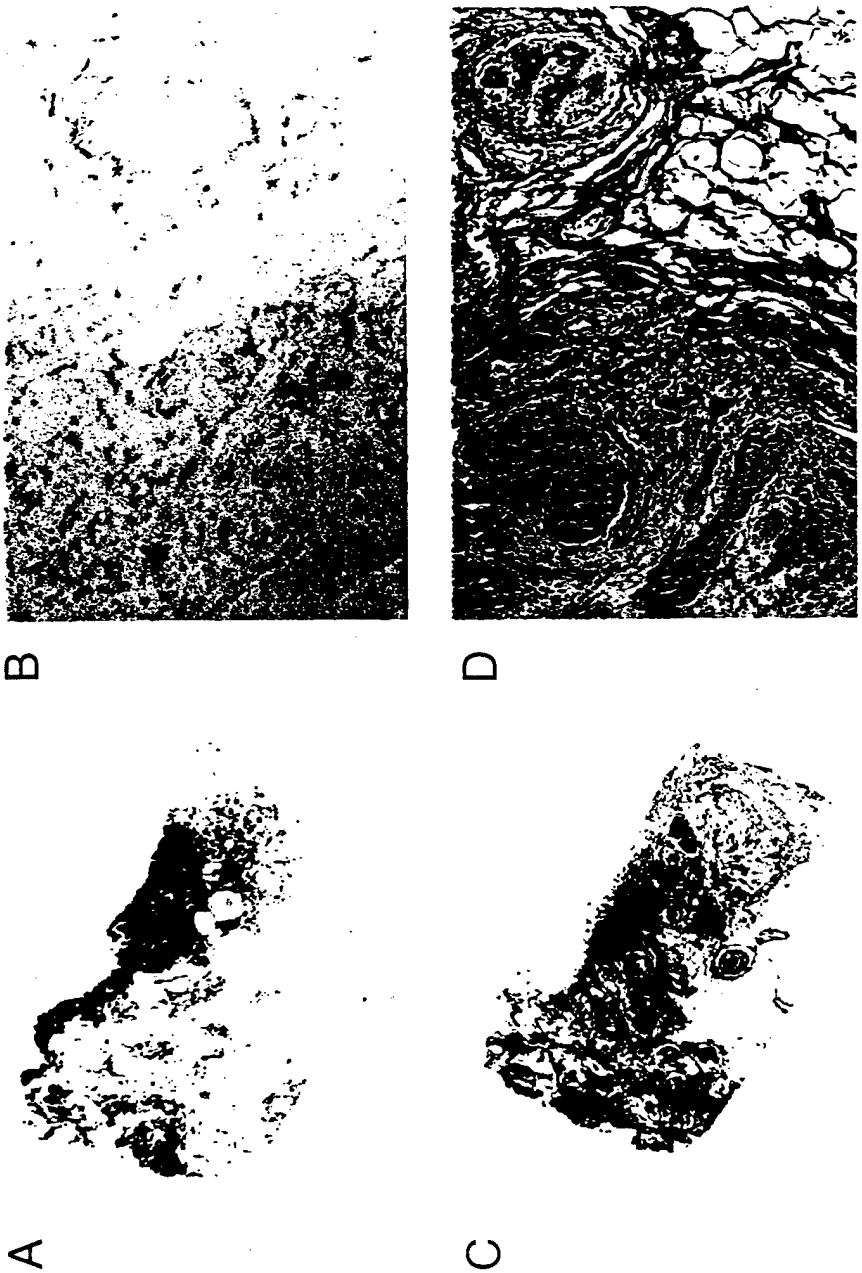


Figure 3

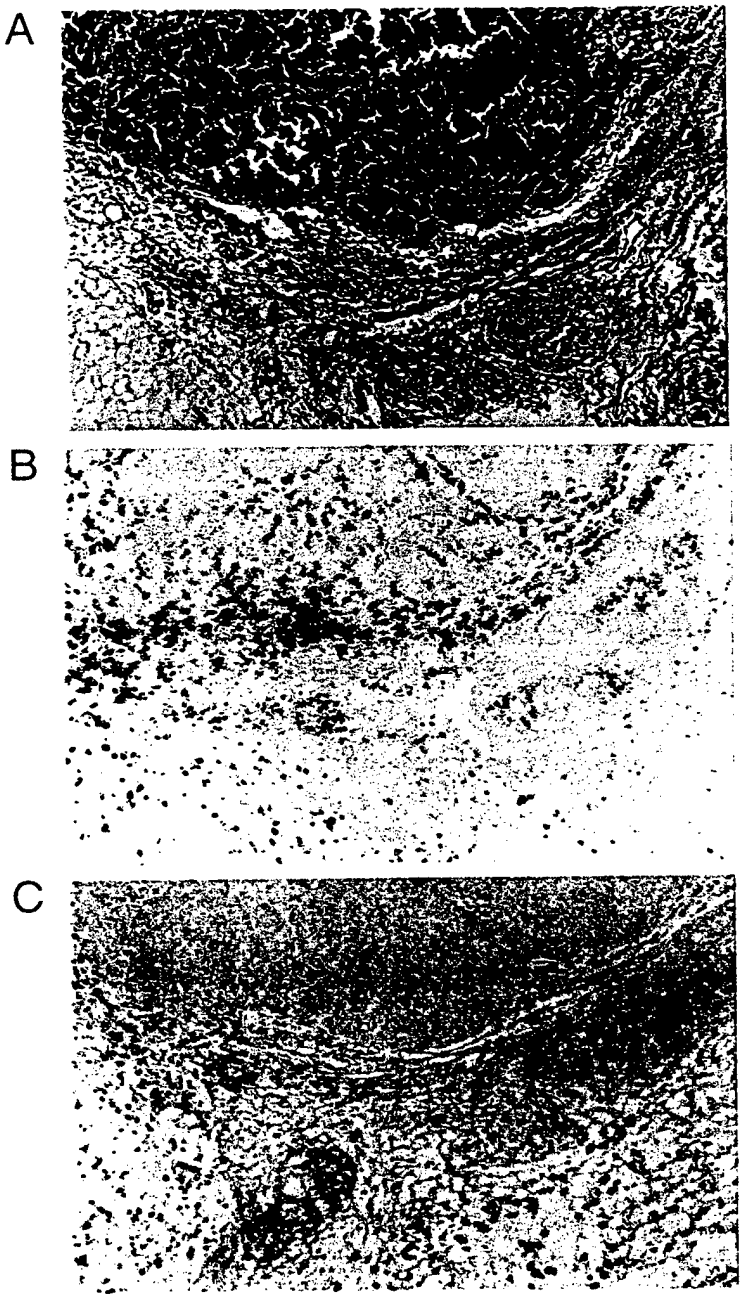
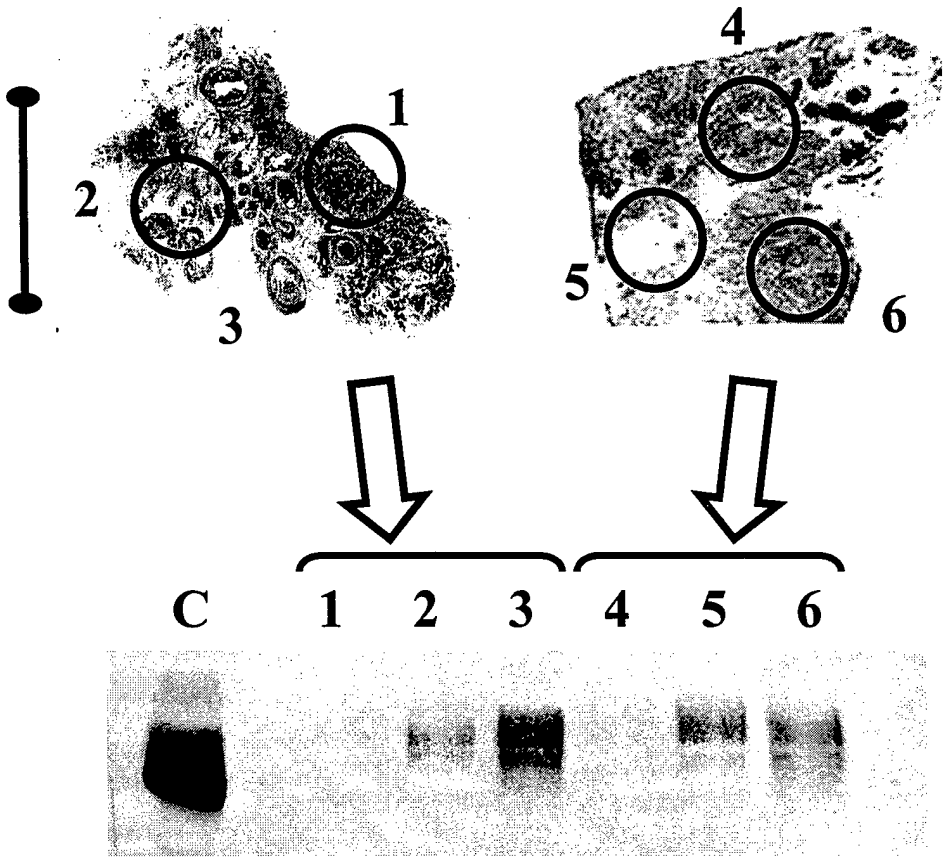


Figure 4



APPENDIX G

MAMMAGLOBIN, A POTENTIAL MARKER OF BREAST CANCER MICROMETASTASIS[1]

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Running title: *Mammaglobin* expression in breast cancer.

Key words: *Mammaglobin*, uteroglobin, breast cancer, tumor progression, metastasis.

Footnotes:

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[3] The abbreviations used are: RT-PCR, reverse transcription-polymerase chain reaction; H&E, Hematoxylin/Eosin; ER, estrogen receptor; PR, progesterone receptor; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

[a] N°: case number; For each case, *mammaglobin* score (cells estimated average intensity x proportion of positive), determined as indicated in "Materials and Methods", is given for normal, *in situ*, invasive and when available in corresponding axillary lymph node. na: not available. NS: axillary nodal status.

Abstract

The *mammaglobin* gene is overexpressed in breast tumors but the cellular origin and relationship to tumor progression is unknown. We found *mammaglobin* overexpressed in the *in situ* compared to the invasive component within an individual human breast tumor. *In situ* hybridization analysis of 13 primary tumors containing normal, *in situ*, and invasive elements, and in some cases (9/13) axillary lymph node metastases, revealed that *mammaglobin* expression occurs in all compartments, is restricted to epithelial cells, and is increased in tumor cells compared to normal ones ($p<0.04$). Reverse transcription-PCR analysis of 20 tumors and matched lymph nodes showed a direct correlation ($p=0.0001$) between *mammaglobin* mRNA expression and histological detection of nodal metastases. Our results suggest that *mammaglobin* could be a marker of axillary lymph node breast metastases.

Introduction

Detection of breast cancer micrometastases based on specific genetic markers may provide useful information to guide early therapeutic decisions (1). Various biological markers have been proposed for the detection of breast cancer cells, including *Keratin*-19 and *Muc*-1 (2). However the frequency of expression of these markers is often related to tumor differentiation and is not always confined to breast tissue (3). This relates in part to the fact that most potential markers are derived initially from the study of other tissues and systems.

In order to better understand the molecular alterations that are specifically involved in breast tumor progression and that might provide molecular targets for detection, we and others have undertaken direct studies of human breast tumors. These studies aim to identify genes differentially expressed between normal and neoplastic tissue or between different components of the same human breast tumor (4-6). *Mammaglobin*, a member of the uteroglobin gene family, has recently been identified by this approach as a gene that is overexpressed in breast tumors (7). In this study we have also identified *mammaglobin* mRNA as being differentially expressed between the non-invasive *in situ* and invasive components of a single breast tumor. We have gone on to investigate the cellular origin *in vivo* and the pattern of expression of *mammaglobin* mRNA in relation to tumor progession within primary tumors and their nodal metastases by *in situ* hybridization and reverse transcription-polymerase chain reaction (RT-PCR).[3]

Materials and methods

Human breast tissues and cell lines

All breast tumor cases, that have been processed as previously described to create paraffin embedded tissue blocks and mirror image frozen tissue blocks (8), were selected from the NCIC-Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). Four groups of tissues or cell lines were used in this study as detailed below.

The initial "microdissection case" consisted of a single tumor (Tumor N° 2, Table 1) with extensive *in situ* lobular and limited (<25%) invasive lobular components.

For the *in situ* hydridization studies, 13 tumors were selected that contained normal ducts/lobules, *in situ* and invasive tumor components within a single section. For 9 of these cases, axillary lymph node metastases were available and analyzed simultaneously. For each of these tumors, the nodal status, i.e absence (lymph node negative) or presence (lymph node positive) of metastasis within corresponding axillary lymph nodes had been determined by histologic analysis of H&E stained lymph node sections (Table 1).

For RT-PCR analysis of *mammaglobin* expression in primary breast tumors and their corresponding axillary nodes a second panel of 20 different patients, was selected. This panel consisted of 13 node positive tumors and seven node negative tumors, as determined by histologic examination of node sections.

For study of cell lines, both ER positive and negative breast cancer cells (T47-D, T47-D5, MCF-7, ZR-75, HBL-100, MDA-MB-231) were grown as previously described (9). Total RNA was extracted from frozen tissue sections or cell lines with Tri-reagent (MRCA, Cincinnati, OH) according to the manufacturer's instructions.

Subtractive hybridization analysis

The microdissection of the initial single tumor case was performed as previously described (8). After extraction of total RNA, the subtractive hybridization was performed as previously described, using the *in situ* and invasive component of the initial microdissection case as the tester and the driver, respectively (4).

In situ hybridization

Paraffin embedded 5 µm breast tumor sections were analyzed by *in situ* hybridization according to a previously described protocol (5). The plasmid Mam-503, that consisted of PCR™II plasmid (Invitrogen, SanDiego, CA), containing a 503 bp insert of *mammaglobin* cDNA, between bases 1 and 503 (7), was used as a template to generate [^{35}S] UTP-labeled sense and antisense riboprobes using Riboprobe® Systems (Promega, Madison,

WI) according to the manufacturer's instructions. Sections were then developed and counterstained with H&E after 15 days. Levels of *mammaglobin* expression were assessed in normal, *in situ*, invasive and when possible nodal metastastic regions by brightfield microscopic examination at low power magnification and using a previously described semiquantitative approach (10). Scores were obtained for each component by estimating average signal intensity (on a scale of 0 to 3) and the proportion of epithelial cells showing a positive signal (0, none; 0.1, less than one tenth; 0.5, less than one half; 1.0 greater than one half). The intensity and proportion scores were then multiplied to give an overall score. Statistical comparisons between matched compartments have been performed using the two-tailed Wilcoxon signed rank test. The correlation between *mammaglobin* expression within the invasive component and the corresponding nodal metastasis has been established by calculation of the Spearman rank correlation coefficient.

RT-PCR analysis

One µg of total RNA was reverse transcribed in a final volume of 20 µl and one µl of the reaction mixture subsequently amplified by PCR as previously described (5). Primers used corresponded to *mammaglobin* (sense 5'-CCGACAGCAGCAGCCTCAC-3', located in *mammaglobin* sequence between bases 41-59, and antisense 5'-TCCGTAGTTGGTTTCTCAC-3', located in *mammaglobin* sequence between bases 401-383 (7) and to the ubiquitously expressed glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene (sense 5'-ACCCACTCCTCCACCTTTG-3' and antisense 5'-CTCTTGTGCTCTTGCTGGG-3'). To amplify cDNA corresponding to *mammaglobin* and *GAPDH*, 30 cycles (30 seconds at 94°C, 30 seconds at 55°C and 30 seconds at 72°C) of PCR were used. Ten microliters of *mammaglobin* PCR and *GAPDH* PCR were mixed and analyzed by electrophoresis on prestained (ethidium bromide, 15 µg/ml) 2% agarose gels. Identity of the expected 361 bp long fragment corresponding to *mammaglobin* was confirmed by sequencing. Association between *mammaglobin* expression within nodal metastasis and histopathological determination of nodal status was tested using the Fisher's exact test.

Results

Identification of mammaglobin mRNA in breast cancer.

A "microdissection case" that contained lobular carcinoma *in situ* associated with invasive carcinoma was selected and frozen tissue blocks were subjected to microdissection to obtain material for extraction of total RNA corresponding to both regions (8). This RNA provided the substrate for a recently described subtractive

hybridization technique (4). Upon completion of subtraction a 503 bp long fragment was isolated as corresponding to a gene overexpressed in the *in situ* compartment (data not shown). Sequencing of this fragment identified nucleotides 1 to 503 of the sequence encoding *mammaglobin*, a recently described putative member of the uteroglobin family (7).

Assessment of mammaglobin mRNA expression in normal breast tissue, in situ and invasive tumor elements and corresponding axillary lymph nodes.

To establish which cells express *mammaglobin* mRNA within breast tumor tissues and to examine the distribution of the expression of this mRNA within different breast tumor components, 13 cases that included both lobular and ductal tumors were selected from the NCIC-Manitoba Breast Tumor Bank. For each case, age of the patient and clinical characteristics of the tumor (i.e Nottingham grade, ER and PR levels, and nodal status) are given in Table 1. Paraffin tissue sections containing normal duct/lobules, *in situ* and invasive elements were studied by *in situ* hybridization, together (when available) with corresponding axillary lymph node paraffin sections (Fig. 1). No signal was detectable when using a sense probe (Fig. 1A). In contrast, a signal varying in intensity was observed in epithelial cells when using an antisense probe (Fig. 1B-D). *Mammaglobin* mRNA was not detected in stromal or inflammatory cells in any of the sections studied. Expression of *mammaglobin* was quantified in each component using a semi-quantitative approach described in the "Materials and Methods" section (Table 1). Although *mammaglobin* mRNA was found to be overexpressed in the *in situ* tumor cells compared to normal adjacent epithelial cells in 7/13 cases, this difference did not reach statistical significance (Wilcoxon signed rank test, $n=9$, $p>0.05$). Similarly, even though *mammaglobin* expression appeared higher within the invasive component compared to normal adjacent elements in 6/13 cases, this difference was not statistically significant (Wilcoxon signed rank test, $n=10$, $p>0.05$). However, when both *in situ* and invasive elements were combined, *mammaglobin* expression observed in tumor was higher than that seen in normal adjacent cells (Wilcoxon signed rank test, $n=12$, $p<0.02$). This suggests that although *mammaglobin* mRNA is predominantly overexpressed in cancer cells within the primary tumor, the exact stage at which this increase in expression occurs varies between tumors and the nature of the alteration is complex. For example, when comparing matched *in situ* and invasive components, *mammaglobin* mRNA level was found increased within *in situ* elements in 5/13 (40%) cases (Table 1, cases 1-5) but was increased within invasive elements in 5/13 (40%) cases (Table 1, cases 6-10) and was similar and low in 3/13 (20%) cases (Table 1, cases 11-13).

Interestingly, in 7 out of 9 cases where frozen tissue sections from nodal metastasis were available, *mammaglobin* expression was also detectable within metastastic tumor cells (Table 1). This detection was possible in both early metastasis within the subcapsular sinus (Fig. 1C) as well as established metastasis (Fig. 1D). Evaluation of *mammaglobin* expression within these lymph node metastases revealed a significantly (Wilcoxon signed rank test, $n=8$, $p<0.04$) higher level of *mammaglobin* mRNA than that detected in matched normal tissue.

Interestingly, the level of expression of *mammaglobin* mRNA within axillary lymph node metastatic cells appeared strongly correlated to the level observed within matched invasive elements (Spearman $r=0.89$, $p<0.002$). *Mammaglobin* mRNA level in the primary tumor component appeared unrelated to tumor type, grade or ER/PR levels.

Analysis of mammaglobin mRNA expression in primary breast tumors and their corresponding axillary nodes.

Because it was possible to detect *mammaglobin* mRNA in axillary lymph node metastases in 7 out of 9 metastases by *in situ* hybridization, and because this mRNA had previously been described as being absent from normal lymph node tissue (7), we investigated the possibility that *mammaglobin* could be a marker of axillary lymph node metastases. Twenty independent cases were selected, 13 axillary lymph node positive and 7 node negative. Total RNA was extracted from frozen primary tumor sections and frozen node sections of corresponding axillary lymph nodes. The histological status of all tissues was confirmed in paraffin sections cut from adjacent mirror image paraffin tissue blocks that had been processed in parallel to the frozen blocks (8). Total RNA was reverse-transcribed and analyzed by RT-PCR using primers recognizing specifically *mammaglobin* cDNA and chosen to span intronic regions. PCRs were performed in triplicate with reproducible results. Identity of the PCR product obtained has been confirmed by sequencing. As shown in Figure 2, no *mammaglobin* expression was detected in lymph nodes from cases without histologically detectable tumor cells (0/7 cases). In contrast, all lymph nodes previously identified to contain metastatic breast tumor cells following histopathological assessment had detectable *mammaglobin* expression (13/13 cases). The RT-PCR detection of *mammaglobin* mRNA in axillary lymph nodes appeared therefore strongly associated (Fisher's exact test, $p<0.0001$) with the histopathological detection of lymph node metastases. Interestingly, *mammaglobin* mRNA was detected at a higher frequency in node positive primary tumors (12/13 cases, 90%) than in node negative

primary tumors (4/7 cases, 60%). The absence of signal was unlikely due to degraded RNA and/or cDNA as shown by the amplification of the ubiquitously expressed *GAPDH* cDNA in the same cDNA samples.

Mammaglobin mRNA expression in human breast tissue cell lines.

Mammaglobin mRNA expression was also investigated in different human breast epithelial and cancer cell lines. Total RNA was extracted from T47-D, T47-D5, MCF-7, MDA-MB-231, HBL-100, ZR-75 cells and analyzed by RT-PCR. *Mammaglobin* mRNA was detectable only in the ZR-75 breast cancer cell line (data not shown).

Discussion

Mammaglobin has recently been identified as a mammary specific gene overexpressed by approximately 23% of primary breast tumors (7). *Mammaglobin* is considered to be a member of the uteroglobin gene family and also maps to a region (11), 11q13 that is frequently associated with alterations in breast tumorigenesis (12-18). However the cellular origin of expression of *mammaglobin* and the relationship between expression and tumor progression has not been previously determined. Our results show that the pattern of *mammaglobin* expression within breast tissues and tumor components is complex. However expression is restricted to mammary epithelial cells and in those tumors that show overexpression of *mammaglobin* in the invasive primary tumor, this expression is invariably conserved in the concurrent axillary nodal metastases.

Mammaglobin was identified in the present study through detailed analysis of a single breast tumor to distinguish genes differentially expressed at early stages of tumor progression. The initial observation suggested that *mammaglobin* might be more highly expressed in the pre-invasive *in situ* than in the invasive elements of breast tumors. This is a pattern of expression that would be consistent with data that suggests that expression of the uteroglobin can influence the invasiveness of epithelial tumor cells in other tissues (19, 20). However, subsequent analysis revealed a more complex pattern of expression. In some cases (≈20%) expression was undetectable by *in situ* hybridization at all stages, in some (≈40%) the expression changed little or even declined with progression from *in situ* to invasive disease and in others (≈40%) the expression increased. We also found that *mammaglobin* expression in the primary tumors was unrelated to tumor type, grade, or steroid receptor levels as has been previously reported (7, 11).

This complexity in expression may be related to the fact that the *mammaglobin* gene has been recently localized to a chromosomal region, 11q13 (11), that has been previously shown to be commonly modified during breast

tumorigenesis. Alterations at this locus include loss of heterozygosity at markers adjacent to and on either side of the *mammaglobin* gene (*Pygm* and *Int*-2) occuring at increasing frequencies (from 9% up to 67% of informative cases) in association with progression from atypical ductal hyperplastic breast lesions, lobular and ductal *in situ* neoplasic lesions to invasive breast cancer (12, 13, 18). Amplification of the 11q13 region that includes several known genes such as *int*-2, *cyclin* D1 and *ems*-1, has also been observed in up to 15% of invasive breast carcinoma (14-17) although the amplified region may not directly involve the *mammaglobin* gene. Therefore the possible role of 11q13 modifications on differential expression of the *mammaglobin* gene between normal and cancer breast tissue remains to be addressed.

In the course of our own molecular studies, even though based on microdissected tissues, we have identified genes expressed only by epithelial cells (5), only by fibroblastic cells (10) or by both tumor and stromal cell types (our unpublished data). Therefore, the determination of the epithelial cellular origin and range of tumor components that express a particular gene *in vivo*, as opposed to within established cell lines, is of crucial importance to understand the molecular functions of encoded proteins and the potential application as a tumor cell marker.

Given the previous suggestion (7) that *mammaglobin* expression is restricted to breast tissues we have examined the potential of this gene to be a marker of breast metastasis within axillary lymph nodes. Using RT-PCR, it was possible to detect *mammaglobin* in 13 out of 13 sections from axillary lymph nodes shown by histopathological examination of adjacent paraffin sections to contain metastatic cells and in none of 7 nodes that were negative. The increased frequency of detection of *mammaglobin* in breast tumor cells using RT-PCR (primary 16/20 cases, 80%; metastasis 13/13 cases, 100%) compared to *in situ* hybridization (primary 10/13 cases, 77%; metastasis 7/9 cases, 77%) or Northern blot (primary 23%) as used by others (7), is most likely due to differences in sensitivity between assays.

The physiological role of *mammaglobin* in breast tissue as well as its possible involvement in breast tumorigenesis is unknown. Our data suggest however that *mammaglobin* gene expression is modified during breast tumorigenesis and may be a good candidate marker for the detection and characterization of breast cancer metastasis.

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No	Mammaglobin score: normal ducts/lobules	Mammaglobin score: in-situ carcinoma	Mammaglobin score: invasive carcinoma	Mammaglobin score: nodal metastasis	Tumor characteristic: Tumor Type	Age years	Grade	ER	PR	NS
1	**0.0** (0.0x0.0)	**0.1** (1.0x0.1)	**0.0** (0.0x0.0)	**0.1** (1.0x0.1)	Ductal NOS	56	6	47	4.9	+
2	**0.2** (2.0x0.1)	**1.5** (3.0x0.5)	**0.0** (0.0x0.0)	**1.0** (2.0x0.5)	Lobular	45	7	3.5	28	+
3	**0.1** (1.0x0.1)	**0.2** (2.0x0.1)	**0.0** (0.0x0.0)	**na**	Lobular	75	5	7.4	9.1	-
4	**0.1** (1.0x0.1)	**0.5** (1.0x0.5)	**0.1** (1.0x0.1)	**0.2** (2.0x0.1)	Ductal NOS	48	8	0.6	2.2	+
5	**0.2** (2.0x0.1)	**2.0** (2.0x1.0)	**1.0** (1.0x1.0)	**2.0** (2.0x1.0)	Lobular	58	6	37	15.9	+
6	**0.1** (1.0x0.1)	**0.1** (1.0x0.1)	**1.0** (2.0x0.5)	**1.5** (3.0x0.5)	Ductal NOS	63	7	1.8	12.2	+
7	**0.2** (2.0x0.1)	**0.2** (2.0x0.1)	**2.0** (2.0x1.0)	**na**	Lobular	70	5	9.2	53	-
8	**0.0** (1.0x0.0)	**2.0** (2.0x1.0)	**3.0** (3.0x1.0)	**3.0** (3.0x1.0)	Lobular	48	5	10.3	7.2	+
9	**0.0** (0.0x0.1)	**2.0** (2.0x1.0)	**3.0** (3.0x1.0)	**na**	Ductal NOS	72	8	32	46	-
10	**0.1** (1.0x0.1)	**0.1** (1.0x0.1)	**3.0** (3.0x1.0)	**2.0** (2.0x1.0)	Duct-Lob mix	44	6	0.6	27	+
11	**0.0** (0.0x0.0)	**0.0** (0.0x0.0)	**0.0** (0.0x0.0)	**0.0** (0.0x0.0)	Ductal NOS	80	7	1	8.5	+
12	**0.5** (1.0x0.5)	**0.0** (0.0x0.0)	**0.0** (0.0x0.0)	**0.0** (0.0x0.0)	Ductal NOS	36	7	22	42	+
13	**0.2** (2.0x0.1)	**0.0** (0.0x0.0)	**0.0** (0.0x0.0)	**na**	Ductal NOS	32	7	5.8	41	+

Table legend

Table 1

Clinical and pathological features of breast tumors studied for *mammaglobin* mRNA expression by *in situ* hybridization.[a]

Figures legend

Fig. 1

Expression of *mammaglobin* mRNA in a primary breast tumor and concurrent nodal metastases (case number 10, Table 1) studied by *in situ* hybridization. Plates A-B illustrate consecutive sections from the primary tumor showing a normal lobular unit (in the middle) surrounded by invasive lobular carcinoma. Plate C shows a focus of early metastatic tumor combined to the subcapsular lymph node sinus. Plate D shows established nodal metastasis in an adjacent lymph node. A: sense probe; B, C, D: antisense probe.

Fig. 2

RT-PCR analysis of *mammaglobin* and *GAPDH* mRNA expression in primary breast tumors (P) and their corresponding axillary lymph nodes (N), histologically shown to contain (Node positive) or not contain (Node negative) metastases. PCR products were mixed before separation on 2% agarose gels prestained with ethidium bromide. Black arrow: product corresponding to *mammaglobin*, grey arrow: product corresponding to *GAPDH*. M: Molecular weight marker (ϕx174 RF DNA/Hae III fragments, Gibco BRL, Grand Island, NY). N: Negative control, no cDNA added during the PCR reaction.

Figure 1

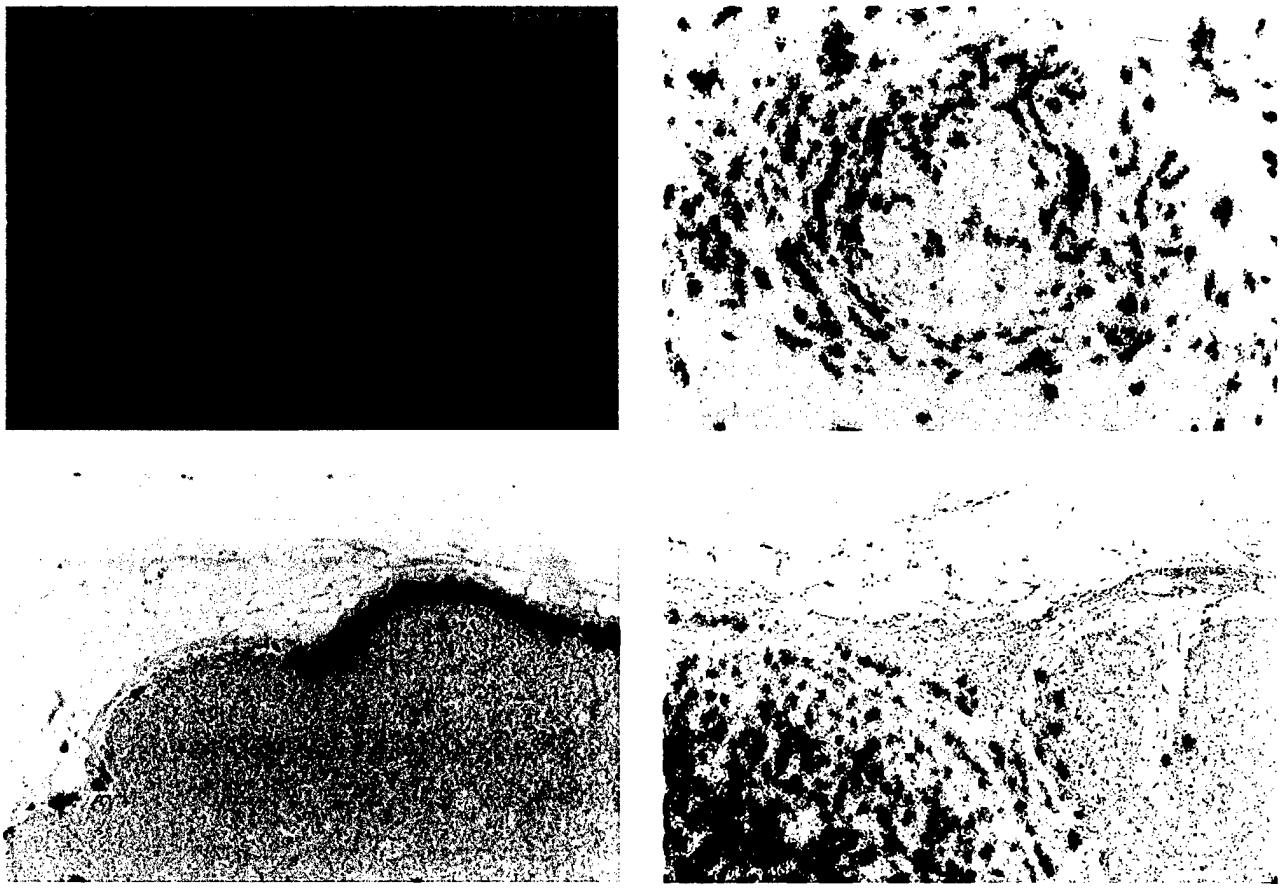
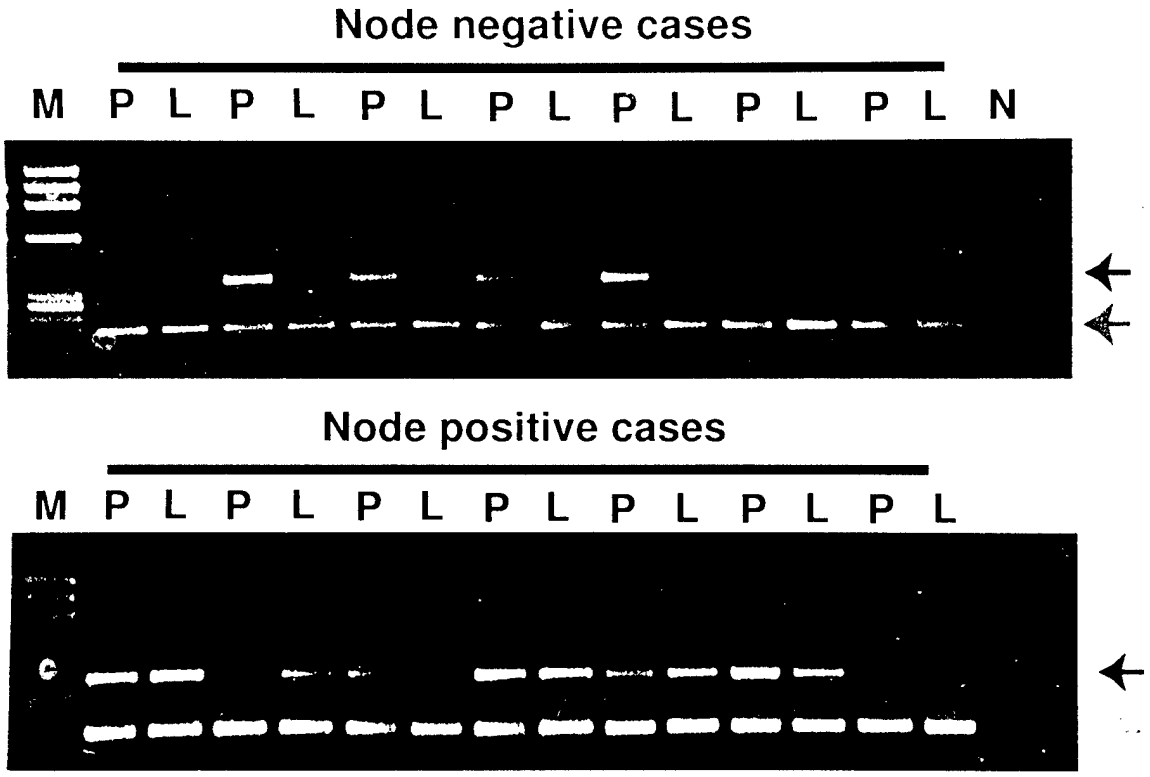


Figure 2


Node negative cases
M P L P L P L P L P L P L P L N
Node positive cases
M P L P L P L P L P L P L P L